SPREADING OF INACTIVATION IN AN UNBALANCED X-AUTOSOME TRANSLOCATION

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The phenomenon of the spreading of gene inactivation from X chromatin into adjacent autosomal loci in mammalian X-autosome translocations has interested many investigators. A major motivation for this interest is that this system is, in many respects, a simplified model for the control of genetic expression in general. It is well known that the expression of large groups of genes is suppressed in differentiated cells of multicellular organisms, but the mechanisms involved are poorly understood. Studies of spreading of inactivation are useful because they can provide answers to specific questions regarding at least one type of systematic genetic suppression. Once the properties of translocated X segments necessary for the initiation of spreading are clarified, we will know more about the functional organization of the X -- whether certain loci are essential and, if so, whether these loci contain sub-loci with specialized functions, which X regions require continuity and/or a given polarity in order to work properly, and whether all segments capable of inducing suppression do it in the same way. We will know, therefore, more about centers of organization for the suppression of large groups of genes. Once the rules governing the ability of an autosomal segment to permit spreading and the rules governing termination

are clarified, we will know more about how large numbers of genes are suppressed as functional units -- what constitute termination signals and how efficient these signals are, whether suppression travels differently through different "classes" of genes (eg. structural genes, "housekeeping" genes, repetitive sequences), and whether areas can be "skipped". Thus, studies of this kind promise to bear fruit in terms of a better understanding of processes fundamental to gene control.

The first section of this introduction will give a general overview of the topics of inactivation in the X chromosome and inactivation in X-autosome translocations. The second section will deal specifically with spreading of inactivation in X-autosome translocations.

X INACTIVATION IN THE X CHROMOSOME AND IN X-AUTOSOME TRANSLOCATIONS

The first insights into X chromosome inactivation came in the 1920's with the realization that in Drosophila melanogaster, females homozygous for certain X-linked genes had the same phenotype as hemizygous males (1). This was called "dosage compensation" by Muller et. al. (2). The dosage compensation process assures that male somatic cells, receiving one allele per X-linked locus and

female somatic cells, receiving two alleles per X-linked locus have the same level of gene product. According to a review by Gartler and Andina (3), there is evidence that the mechanisms responsible for "dosage compensation" accomplish other ends: they provide for an inactive X in spermatogenesis in the male and for two functional X's in oogenesis in the female. The exact functional significance of these latter features is unclear, but these authors feel that prevention of rearrangement of sex determining genes between the X and Y is involved.

It appears that there are three major means by which dosage compensation is achieved in nature, of which X chromosome inactivation is only one. In the Drosophila melanogaster, most individual X-linked genes are controlled by an X-linked modifier gene (or genes) specific for them. These modifier genes control the level of transcriptional activity for their target gene(s) so as to equalize the rate of gene product formation in males and females (4,5). It is not clear whether there is an increase in X-linked gene activity in XY males to the level resulting from a double gene dose in the female, a reduction in X-linked gene activity in each of the two X's in the female to the level resulting from a single gene dose in the male, or a combination of both these processes. It is known, though, that neither of the two X's in the female is "shut off."

The second mechanism of dosage compensation is seen in the creeping vole (Microtus oregoni). The male of the species is XY and the female is XO. The only viable spermatogonia are OY (produced by non-disjunction or anaphase lag in the primordial germ cell) and the only viable oogonia are XX (also produced by non-disjunction or anaphase lag). Thus, both sexes in this species have an equal complement of X-linked alleles, and the requirements proposed above for X activity in gametogenesis are met.

The last mechanism of dosage compensation, X inactivation, was first eludidated by Mary Lyon (7). In order to explain the mosaic phenotype (colored coat patches) exhibited by mice bearing X-linked mutant genes affecting coat color, she suggested that each color patch is composed of a clone of cells in which one of the two X's, maternal or paternal, is genetically inactive; the inactivated X is the same for each cell in the clone. The cell from which a given clone originated was proposed to have had its X inactivated during embryogenesis. Moreover, during the stage of embryogenesis in which this occurred, each cell of the organism was hypothesized to have had one of the two X chromosomes inactivated, the X affected in any given cell being chosen randomly. Thus, each embryonic cell subsequently gives rise to a clone of cells bearing the same inactivated X making the organism a phenotypic mosaic.

Lyon's hypothesis was given experimental verification by Davidson et. al. in 1963 using skin cell cultures of human females who were heterozygous for the X-linked gene coding for G-6PD. In their experiment, each cultured cell gave rise to a clone of cells producing either one or the other electrophoretic variant of the enzyme but not both (8). One additional important property of X inactivation that has been shown since Lyon's proposal is that a single X is active in a diploid mammalian cell no matter how many supernumerary X's are present (3).

Two features not directly related to gene expression have been found to accompany X-inactivation and have been useful experimental tools. The first is that the inactive X in many mammalian species is morphologically distinguishable in interphase nuclei. This X appears heterochromatic. In the human a specific "body" ("Barr body") exists in the nucleus (9). Proof that the heterochromatic X is indeed the genetically inactive X came with Ohno and Cattanach's experiments with female mice heterozygous for the "flecked" translocation -- an autosomal insertion in the X (10). They showed that the translocation chromosome -- which is morphologically identifiable -- was heterochromatic in a cell if and only if the cell's phenotype indicated that an inserted autosomal gene had been inactivated. Further proof that the heterochromatic X is

the inactive X was provided by Comings who demonstrated that this structure exhibited markedly diminished RNA synthesis as evidenced by low tritiated uridine incorporation (11).

The second feature accompanying X inactivation is DNA replication asynchrony between the inactivated X and the active X plus the autosomes (3). In some animals, such as the house shrew (Suncus Murinus), the inactive X seems to initiate DNA synthesis synchronously with and terminate DNA synthesis earlier than the active X and the autosomes (12). However, the phenomenon of replication asynchrony is often referred to as " late replication" because in most species customarily studied (eg. mouse and man) the inactive X initiates and terminates replication later than the other chromosomes (13,14,15,16). It should be noted that in certain X chromosome rearrangements in the human, the extent of inactivation as judged by replication asynchrony has disagreed with the extent as judged by the patient's phenotype. This has prompted some authors to question, in these circumstances at least, the theory of tight correspondence between inactivated loci and late replicating loci (17,18,19). In general, though, late replication has proven a useful and reliable marker for inactivated chromosomal material.

There have been several systems in which biologists have

studied mammalian X-chromosome inactivation. One of the most useful and productive approaches has been the study of X-autosome translocations. Study of these translocations has been even the more successful because of the variety of translocations that have been found. Over 50 human X-autosome translocations have been reported and every autosome except #10 has been implicated (20).

One of the most striking findings that emerged early from the studies of X-autosomal translocations was that in most of these cases the choice of the inactive "X" (or more properly the choice: which of the various chromosomes bearing X material is (are) to be inactivated?) was not made randomly. This was apparent because of the failure of the organisms to demonstrate equal proportions of cells displaying each conceivable inactivation pattern. Initially, this phenomenon of "preferential X inactivation" was invoked to challenge the Lyon hypothesis (22). However, thereafter, there evolved a consensus of opinion that the X-autosome translocations constituted a separate and distinct system in which X chromosome inactivation could occur and from which one could not loosely extrapolate hypotheses about inactivation in normal karyotypes.

Why is it that a majority of cells in many X-autosome translocations demonstrate a certain inactivation pattern? Aside from the problem of "spreading", this question has been the most impor-

tant one with respect to genetic control suggested by studies of X-autosome translocations. For this reason, it is addressed below in some detail.

Answers to this question began to become evident when investigators looked for consistencies among those karyotypes which demonstrated similar inactivation patterns. It was discovered that the pattern of X inactivation in a given translocation was related to whether or not the translocation was balanced or unbalanced. As a rule, in balanced X-autosomal translocations, almost all cells tested showed inactivation of the "normal" X in preference to the X material present on the translocation chromosomes (23, 24,25). On the other hand, typically, in unbalanced X-autosomal translocations containing a normal X and a derived (X), almost all cells tested showed inactivation of the derived ("abnormal") X (23,26,27). There do occur, however, exceptions to both of the above generalizations. There are balanced X-autosomal translocations which have a substantial proportion of cells exhibiting late replication of the X translocation chromosome (27,28,29) and even "preferential" inactivation of the X translocation chromosome (30, 31). Conversely, there are unbalanced X-autosomal translocations which have a substantial proportion of cells exhibiting late replication of the normal X (32,33) or "preferential" inactivation

of this chromosome (34). Moreover, there exist translocations, both balanced (35,36) and unbalanced (32,37) which have a significant number of cells displaying simultaneous inactivation of both normal and translocation X chromosomes.

In an attempt to explain the above data and the phenomenon of "preferential inactivation" in a wider sense, three major hypotheses have been advanced. The first, advanced by Cohen et. al., and later by Gilgankrantz et. al., suggested that the inactivation pattern depends on the "direction of chromosomal exchange." These authors anticipated that when an X segment is translocated to an autosome, the normal X will be inactivated whereas when an autosomal segment is translocated to an X, the X translocation chromosome will be inactivated (35,38). Cases collected after their proposal was made have provided several clearly contradictory karyotypes (26,27) and as a result, the hypothesis is now considered disproved.

The other two hypotheses, in contrast, are still favorably regarded. One of these -- the "structural" hypothesis -- states that whether or not a translocation chromosome containing X material is inactivated is an inherent structural property of the chromosome itself. The second hypothesis -- the "selection" hypothesis -- states that during embryogenesis X inactivation is initially random. However, it suggests that cell

lines having inactivation patterns with lesser fitness will be selected against; this process will give rise to an organism whose tissues seem to show a "preferential" inactivation pattern. The "selection" hypothesis implies that the greater the discrepancy in fitness, the greater the predominance of one cell line over another.

There are major arguments both for and against each of these theories. Ohno was one of the first to suggest that it was inherently impossible for a balanced X-autosome translocation to exhibit inactivation of the X material on both translocation chromosomes (4). Since then, karyotypes supportive of the structural hypothesis have been found. Mikkelsohn and Dahl report an unbalanced translocation 46,X,der(X), t(X;8)(p22;q21), in which the normal X is inactivated in all cells (34). This arrangement gives a partial monosomy X and partial trisomy 8. It is perhaps conceivable that the respective monosomy and trisomy are not deleterious and hence are not selected against, but it is not conceivable that these imbalances give a selective advantage over the alternative inactivation pattern which provides for a balanced gene dose. Thus, this case seems best explained by a structural predilection of the translocation chromosome not to be inactivated. Similar arguments can be made regarding the balanced karyotypes in the cases of Cohen et. al. (35) and Thelen et. al. (30). One further line of evidence

in favor of the "structural" hypothesis is that nuclei containing chromosomes which possess two long arms of the X often display two Barr bodies (39,40). This implies that there is something inherent to Xq which serves as an "inactivation center " (see next section).

On the other hand, there is one major argument against the theory that the inactivation pattern is a product of the inherent properties of the translocation chromosome(s). This is the fact that the same translocation chromosome in different first degree relatives can show different inactivation patterns depending upon the genetic "environment" (24,28,31,37,41,42,43,44). Further, the alternative hypothesis, cell selection, is supported by the following data. Disteche et. al. have shown that in a certain X-autosome translocation in the mouse, the proportion of cells exhibiting each inactivation pattern changes significantly from a more random pattern to a less random one in going from embryo to adult (45). Another analysis of this mouse translocation examined the proportion of variegated tissue in the animal and this work supported similar conclusions (21). In many human X-autosome translocations, the predominant inactivation pattern is the one which provides for the most balanced expression possible (17,23,24,28,31,46,47); it is felt that a high incidence of such

patterns would be unlikely if the inactivation were based on purely structural features of the translocation chromosomes. Moreover, in the circumstances in which the inactivation pattern allows for unbalanced expression, the unbalanced genotype is very often known to be relatively harmless (18,23,26,28).

At the current time, because neither hypothesis can stand alone, the prevailing feeling is that a combined hypothesis best fits what is known. It is felt that inactivation takes place initially subject to certain constraints based upon the structure of the translocation chromosomes. Following this first step, the relative proportion of cells displaying each inactivation pattern in the adult is determined by selection (26).

Thus, X-autosome translocations have provided insight into some of the requisites for X chromosome inactivation and into modulation of control mechanisms by natural selection at the cellular level.

SPREADING OF X INACTIVATION

Having discussed the selection of the inactive X, we may now turn to the question of the parameters governing spreading of X inactivation beyond the X chromatin.

The study of spreading of inactivation has become ever more sophisticated with the introduction of new methodologies. Data on the subject have been gathered using three principal techniques. The first, and oldest, is the observation of variegated phenotypes; autosomal genes will produce such phenotypes if they are inactivated in some clones of cells (allowing hemizygous expression in these cells) and not in others (where heterozygous expression occurs). These phenotypes obviously occur only in cases in which two (or more) inactivation patterns are present in a significant number of cells. The second technique is the observation of spreading of late replication to autosomal loci -- labels such as H^3-thymidine and BrdU (see below) are introduced late in the S phase of the cell cycle in order to make this possible. The third technique involves enzyme assays for proteins coded for at autosomal loci; enzyme activity should be reduced in proportion to the number of copies of autosomal loci which are inactivated.

The first information relevant to spreading came with the discovery of "position effect variegation" in the Drosophila (48). Drosophila is an organism whose interphase chromosomes are made up of stretches of heterochromatin interspersed with stretches of euchromatin. It was found that in chromosomal rearrangements in which a heterochromatic region is broken and in which a euchromatic

segment is brought into close proximity with the breakpoint, the gene activity on the euchromatic segment can be suppressed. It was also found that the spreading of supression from the heterochromatic break point takes place in a "polarized" manner, i.e. along a gradient. Thus if a certain gene is more distal to the heterochromatic breakpoint than another, the distal gene is inactivated only if the proximal one is. However, the proximal gene may be inactivated independently of the distal one.

After the description of this phenomenon, it was seen that a somewhat analogous situation occurs in the mouse. The heterochromatin making up inactivated segments of X present on X-autosome translocation chromosomes has the power to inactivate attached autosomal loci (21,49). This was apparent because mice bearing certain X-autosome translocations were seen to display a variegated phenotype based on the autosomal loci in question. Mouse studies of this kind clarified several points. They showed that not all translocated X material inactivates nearby autosomal loci, even when the loci are very close to the segment of X. This proved that X material, per se, (with or without an exposed "breakpoint") does not have the power to inactivate autosomal loci. These studies showed, in addition, that whether or not an autosomal locus is inactivated is relatively independent of the distance

of the locus from translocated X material. For instance, there are two X-7 translocations in the mouse (R2 and R3) in which the breakpoints in chromosome 7 are approximately at the same place. Therefore, a particular locus on chromosome 7 -- the pink eye dilution, or p, locus -- is approximately the same distance from translocated X material in each of the two rearrangements. The extent of p variegation in the two translocations is very different however (49). The third point these studies made was that an autosomal segment can be inactivated from either direction, i.e. inactivation can spread to an autosomal locus through either end of the autosomal segment.

Enthusiasm for the investigation of spreading of inactivation in the human grew as it became clear that the phenotypes of many patients were explicable on the basis of inactivation of autosomal loci on their X-autosome translocation chromosomes (as shown by spreading of late replication)(23,27,34,35,43,50,51). For example, Leist et. al. have described a woman with the karyotype 46,X,-X,+der (9), rcp(X;9)(q11,q32) mat (24). The late labeling chromosome is consistently the der(9) and late replication is present on the autosomal portion in almost all labeled cells. From this, it might be expected that the patient would <u>not</u> manifest a partial trisomy 9 because of genetic inactivation of her third copy of

most of 9. This indeed is true. Investigations of this kind have proved that spreading has biological significance and clinical relevance. They also have supported and clarified the "selection hypothesis" described in the previous section.

There is a great variety in the patterns of spreading of inactivation in the described human cases (see Appendix). Autosomes 1,3,6,8,9,14,15,18,21,and 22 have been implicated as having suppressed portions. In some instances in which the X portion of a translocation chromosome is inactive, there is no spread (27,29,31,33,35,52,53,54). In other cases, the entire translocated autosomal segment is inactivated (17,24,27,28,29,30, 31,32,33,56,57). Lastly there do exist several examples in which inactivation seems to have extended some distance into the attached autosomal segment but not to its terminal region (17,20,28,55).

An additional complication is that in some reported cases not all cells from the same individual exhibit the same degree of spreading (17,18,20,28,33,37). For example, Jenkins et. al. have reported a case in which the X portion of X_t is late replicating in 77% of cells while the entire X_t is late replicating in 23% of cells (43). (In this regard, there is some evidence that in those circumstances in which different spreading patterns are possible, there is selection of the cell line carrying the most advantageous

spreading pattern. This was shown by Couturier et. al. who described two sisters who show different spreading patterns in the same translocation chromosome: each pattern provides for the most balanced genetic expression possible in the individual displaying it (17).)

There have been attempts to arrive at some unifying principles to account for the great diversity in patterns of spreading. Some theories have postulated features of translocated X material which are requisites for initiation of spread into an adjacent autosomal segment. In 1974, Therman and Patau proposed that inactivation could spread outward through the short arm of the human X, but not through the long arm (26). This has since been disproved (27,28,55).

A more useful notion has been that of X inactivation center(s). An X inactivation "center", first proposed by Russell (58) in the mouse and Cohen et. al. (35) in man, is said to be a locus on the X chromosome at which inactivation initiates and from which it spreads outward. A segment of translocated X with its surrounding autosomal regions is said to be able to become inactivated only if it possesses an X inactivation center.

If such centers exist, in the case of the mouse there must be at least two. This has been shown with the flecked translocation; Eicher showed that each of two loci in the inserted autosomal segment could be inactivated independently of the other (21); if this

could occur as a result of spread from just one of the two X segments, it would be contrary to the "gradient effect" rule observed in all other X-autosome translocations -- hence the postulation of an inactivation center on each of the X segments. This conclusion has been supported by Disteche et. al. who showed a dissociation between the replication timing in X material distal and proximal to the autosomal segment in "flecked" (45). On the basis of this information and of the spreading patterns observed in a number of X-autosome translocations in the mouse, Eicher has hypothesized that there are several "inactivation units" along the entire length of the mouse X. Each unit is proposed to have an "initiator" and a "terminator" with inactivation spreading in a polarized manner from initiator to terminator. The direction of polarization for all units is not the same. According to her hypothesis, when a unit is split by an X-autosome translocation, inactivation may spread outwards from the initiator until a foreign (autosomal) terminator is recognized. Such terminators are imperfect and sometimes one is not recognized; the inactivation process then continues to another terminator -- hence the variety of spreading patterns. Alternatively, if a rearrangement creates a split between units or creates a split in such a way that inactivation proceeds away from the autosomal segment, the X portion may be inactivated

without any associated spreading. Thus, Eicher's hypothesis attempts to explain spreading behavior by referring to the structures of both X and autosomal portions of the translocation chromosome.

In man, the preponderence of evidence favors one X inactivation center. It is postulated to be in Xq13 just proximal to Xq21 (59). With the exception of two recently published cases (see below) all translocation chromosomes described which had inactive X chromatin contained this portion of proximal Xq (see Appendix). The inference has been that the only reason that one locus would be present in all inactive X fragments is that it is essential for the inactivation. The limit of this "essential" region on the centromeric side has been defined, in part, by the existence of inactive Xq segments lacking parts of proximal Xq (24,55). The distal limit has been defined, in part, by the existence of X translocation chromosomes lacking parts of X distal to proximal Xq (23,31). Further support of the notion of a region in proximal Xq which is essential for inactivation comes with the observation that probably all X deletions, isochromosomes, rings, telocentrics and isodicentrics contain this region (40). It is reasoned that for female cells containing these types of abnormal Xs to have balanced genetic expression and hence be viable, the abnormal X must be inactivated. Since it is to be expected that there will arise a comparable

number of abnormal X's lacking the proposed inactivation center on proximal Xq (eg. iso(Xp), del(proximal Xq)) as those having it, the exclusive survival of the latter class of abnormal X's is presumptive evidence for an area essential for inactivation on proximal Xq. On this assumption, examination of these chromosomes has more precisely delineated the "essential" region.

Cohen, et. al. in 1972 proposed the existence of a second inactivation center located on Xp (35). He cited a reciprocal translocation described by Buckton et. al. (60) 46,X,t(Xp-;14q+). The normal X (X_n) is always late replicating in this case. Cohen reasoned that random inactivation between X_n and the X translocation chromosome (X_t) was prevented by the sequestration of an X inactivation center in the Xp material translocated away from X_t. Karyotypes supporting the existence of a second inactivation center directly were lacking until 1977 when Fraccaro et. al. (29) and Gaal et. al. (33) published cases in which there were late replicating fragments of Xp on autosomal translocation chromosomes. In neither case was there spreading to autosomal regions. There is as yet, no reconciliation between the evidence supporting two (or multiple) and one X inactivation center in the human.

There has been little progress made in the elucidation of properties inherent to autosomal segments which govern permissiveness for spreading of X inactivation. Distance of autosomal loci

from X material, in and of itself, does not seem significant. There has been, for instance, inactivation of segments as large as almost all of human chromosome 9 (24). As previously stated, mouse data support a similar conclusion (49). The centromere seems not to be a barrier to spread (37,33,24). The only autosomal feature proposed to be relevant has been the secondary constriction. Mattei et. al. cited a balanced translocation 46,X, t(9;X)(q12;p22) with the breakpoint on 9 in the region of the secondary constriction (31). The X portion of the X translocation chromosome was consistently inactive but there was no spreading beyond the secondary constriction of 9 into 9q -- hence the possibility of a "blocking" phenomenon.

It is clear, in summary, that there is much to be learned about why spreading takes place when it does and what governs how far it will go. Are there specific "terminators" present in the autosome? If so, how often will each one be recognized: Does spreading in a given autosome end at only a small, finite number of loci, can it end in a broad region surrounding a "terminator" or can it end almost anywhere? Does it have a fixed relation to chromosome bands? What is the role of the secondary constriction? Is there a different tolerance to spread through different types of DNA sequences, eg. repetitive vs. non-repetitive DNA? The

answers to these questions will be helpful in arriving at an understanding of X inactivation and of control of genetic expression. The present study has attempted to answer them in the following way.

An unbalanced X-autosome translocation was chosen in which spreading extends from the X portion of X_t into the autosomal segment partially, but not completely. The one selected was the "KOP-2" translocation discovered by Dr. Pallister in 1967 (61). The prepositus was a 19 year old borderline mentally retarded male with the stigmata of Klinefelter's syndrome. His karyotype is 47,Y, t(X;14)(q13;q32), + der(14), t(X;14)(q13;q32)mat (see fig. 1). This arrangement is thought to have arisen from a nondisjunction at the second meiotic division of his mother's oocyte; the mother carries the balanced form of the translocation. Autoradiography indicates that one of the der(14)'s is late replicating in each cell with spreading of late replication from the X portion into at least 2/3 of the segment of 14 (55). However, the segment of 14 most distal from the X material, comprising about 20% of the entire chromosome, is not late replicating.

The questions cited above were profitably addressed in the KOP-2 translocation. The translocation contains two identical der(14) chromosomes, only one of which is inactivated. For

this reason, a band by band comparison of the two chromosomes for replication asynchrony could be made. The der(14) (which will be henceforth referred to as the "14^X") contains a secondary constriction at approximately the level of the termination of inactivation. The role of the secondary constriction could therefore be examined. Lastly, each 14^X has rRNA genes at the region of autosome 14 most distant from the X derived material. The activity of these genes could be determined separately for each 14^X and in this way it was seen directly whether inactivation had spread into the most distal autosomal loci. Moreover, we were able to look at the effect of the kind of repetitive gene sequence seen in rRNA genes upon inactivation.

Detection of "late replication" was accomplished by terminal labeling with BrdU. BrdU (5-bromodeoxyuridine) is a thymidine analog which quenches the fluorescence of several banding agents when it is incorporated into DNA (47,62). BrdU also has the property of making possible the extraction of chromatin where it has been incorporated (63); it was this property that was exploited in our experiment. BrdU terminal labeling has a much higher resolution than does autoradiography.

Determination of the activity of rRNA genes on the two 14^Xs was made in two ways. Firstly, Ag-staining was performed. Ag-staining of certain proteins closely associated with satellite DNA has been shown to correlate well with activity of rRNA genes

as opposed to mere presence (64,65). Secondly satellite associations were scored. Frequency of satellite associations of acrocentric chromosomes has been shown to be related to the activity of their rDNA (65). The 14^{X}s are identical and therefore were expected to show equal silver staining and equal frequency of satellite association if their rRNA genes were equally active.

In summary, this unbalanced human translocation was chosen as a means of learning about the termination of spread of X inactivation within an adjacent autosomal segment.

METHODS

METHODS OF CULTURE AND STAINING

Cell Culture:

Fibroblast cultures of GM-74 (KOP-2) were obtained at the twelfth passage from the Human Genetic Mutant Cell Repository. Non-synchronized cultures were grown in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum.

Chromosome Preparation:

Loosely attached mitotic cells from subconfluent cultures were collected by pipetting medium several times over the monolayer of cells. The cells were then collected into a 15 ml. centrifuge tube. 0.1 ml. of (10 ug./ml.) colcemid was added to the tube and the tube was incubated for 10 minutes at 37 degrees C. The centrifuge tube was then centrifuged for 7 minutes at 800 RPM. The supernatant was discarded and the cell pellet was resuspended in 10 ml. of 0.075M hypotonic KCl solution for 10 minutes at room temperature. The cells were then centrifuged for 7 minutes at 800 RPM. The supernatant was again discarded and the cells were fixed in freshly prepared methanol-acetic acid mixture (3:1, v/v). After one hour, two changes of fixative were made. Finally, the cells were resuspended in 0.5 ml. of fixative. The cell suspension was

dropped on cold wet slides and allowed to air dry.

Quinacrine Banding:

For Q banding (69), slides were stained for 7 minutes in an aqueous solution of quinacrine mustard (50ug./ml.), rinsed vigorously in running tap water for 2 minutes, mounted in Trismaleate buffer (pH 5.6) and viewed under epi-illumination fluorescence optics using the Zeiss microscope. The filter combination used was BG 38 and AL 546 exciter filters, TK 495 Dichroic mirror, and K 510-530 barrier filters. Metaphases were photographed on H and W Control film. The film was developed in H and W developer.

Silver Staining for Ag-NORs:

The Ag-As method was used for staining (68). Coverslips were removed from the already examined quinacrine stained slides. 3 to 5 drops of 50% (w/v) aqueous silver nitrate were placed on the slide and covered with a coverslip. The slide was then placed in an oven at 55-60 degrees C for 15 minutes. After removing the coverslip, the slide was rinsed in distilled water. Three drops of 3% formalin (v/v; initially titrated to pH 7 with sodium acetate crystals and then to pH 4.5 using formic acid) and 2 drops of ammoniacal silver (prepared by adding 4.5 g of silver nitrate to 5 ml. of concentrated ammonium hydroxide and 7.5 ml. of distilled water) were mixed on the slide and covered with a coverslip.

Development of Ag-NOR's was monitored under bright field illumination. The staining was stopped by rinsing in distilled water and the slide was allowed to air dry. The metaphase spreads previously photographed for Q-banding were located and photographed on High Contrast Copy film. The film was developed in Acufine.

DNA replication patterns:

To study the replication patterns, two protocols were used (63). In the B-Pulse, BrdU was added to the cultures at a concentration of 15 ug./ml. for the last nine hours of the culture. For the T-Pulse, cultures were grown in the presence of BrdU (15 ug./ml.) for 16 hours. They were then rinsed with Ham's F-10 medium containing 6×10^{-4} M thymidine and cultured in the thymidine medium for an additional nine hours. In both cases, 0.1 ml. (10 ug./ml.) colcemid was added to the cultures for $1\frac{1}{2}$ hours before harvest. Mitotic cells were collected by trypsinization. Air dried slides were prepared as above.

Slides were stained in an aqueous solution of Hoechst 33258 (2.5 ug./ml.) for 15 minutes, rinsed briefly in water and mounted in Mc Ilvane's phosphate:citrate buffer (pH7.5). The slides were then exposed to U.V. light from a germicidal lamp at a distance of 5 cm. for 45 minutes. After removing the coverslips, the slides were incubated for 40 minutes in 2xSSC at 60 degrees C, stained

for 10 minutes in 4% Giemsa made in phosphate:citrate buffer (pH 6.5), rinsed in running tap water and air dried. Suitable metaphase spreads were photographed on High Contrast Copy film. The film was developed in Acufine.

METHODS OF ANALYSIS

T Pulse:

A qualitative and quantitative assessment of the staining intensity of the 14 and the two 14^X chromosomes was made. First, the 14^X with the late replicating X segment was distinguished from the 14^X with the early replicating X segment on the basis of the banding pattern of the X segment. The 14^X containing the early replicating X segment was identified by the intense staining of band Xq21 while the 14^X containing the late replicating X segment was identified by the intense staining of bands Xq25 and Xq27 (63).

A total of 36 informative cells were analyzed. These were arranged in a series depending on the time of incorporation of BrdU in the S phase of the cell cycle as reflected by the variation in the banding pattern on chromosome 1.

Lastly, the intensity of staining on the 14, the 14^X containing

the early replicating X segment ($14^{X}E$), and the 14^{X} containing the late replicating X segment ($14^{X}L$) was rated visually. The degree of staining was assessed, individually, for a number of bands on the autosomal portion of these chromosomes. The bands scored for staining intensity were these: 14q31, 14q23, 14q21, 14q12, and 14p1. When a band was not clearly identifiable on a particular chromosome, its position and size were estimated using the other bands on that chromosome and using the bands on the cell's two other 14-bearing chromosomes. Use of the standard Paris Conference nomenclature was deemed appropriate because it has been previously shown that the "replication bands" seen in BrdU studies of replication kinetics correspond well to the bands resulting from treatment with quinacrine mustard and with acridine orange (66). Each band on each chromosome was rated for staining intensity on a scale of 0 to 3 (0 being very pale, 3 being very dark). While each region was being rated, all other areas of the chromosome were masked.

B Pulse:

For cells exposed to the B Pulse a band by band assessment of staining intensity analagous to that performed in the analysis of the T pulse was made. Sixteen informative cells were analyzed. The 14^{X} containing the early replicating X segment (the $14^{X}E$) was distinguished from the 14^{X} containing the

late replicating X segment (the $14^{X}L$); the criterion used in the T Pulse was again employed recognizing that late replicating regions stain palely, not darkly under the B pulse protocol. No assignment of cells to a series based upon the staining of a reference chromosome (such as chromosome #1) was made. The bands scored for staining intensity were those examined in the T pulse. In addition, band 14q11 was analyzed. 14q11 is a quinacrine-dull band in contrast to the aforementioned bands which stain quinacrine-bright. Each band on each chromosome was rated for intensity on the same 0-3 scale used for the T pulse using an analogous masking procedure.

Ag stain:

The silver staining regions of each of the acrocentric chromosomes were classified according to the size of the Ag-NOR on a scale of 0-3 (see D.A. Miller et. al. (65)). In this scale, 0 = absent, 1 = small, 2 = moderate, and 3 = large. Acrocentrics were identified by the quinacrine photographs of the appropriate metaphases. Poorly staining cells were omitted.

Satellite Association:

Satellite associations of the acrocentric chromosomes were scored in all photographed metaphases used in this study. An association was said to occur when the satellite regions of two acrocentric chromosomes were directed towards one another and

were less than one chromatid's width apart. In an "open" ring of associations (each chromosome was associated with two adjacent chromosomes) the associations with each of the two adjacent chromosomes was scored. In a "tight"ring of associations (all chromosomes were associated with all other chromosomes in the ring) the association of each chromosome with each of the others in the ring was scored (65). In the metaphases in which the $14^{X}E$ could be distinguished from the $14^{X}L$, associations were assigned to the specific chromosomes involved. In metaphases in which the two $14^{X}s$ could not be distinguished (eg. Q-banded and Ag stained metaphases), the data were pooled.

RESULTS

A Quinacrine banded karyotype of KOP-2 is shown in figure 1.

T Pulse:

An example of a metaphase photographed as part of the T pulse protocol is shown in figure 2, and an example of the B pulse is shown in figure 3.

As stated in the previous section, the approximate time in the cell cycle at which thymidine was introduced in a given cell was determined by the banding seen on chromosome 1. Figure 4 shows examples of four different labeling patterns of the #1 chromosome, reflecting exposure to thymidine at four different times in the S phase of the cell cycle. In the T pulse protocol, the presence of thymidine causes actively replicating chromosomal regions to stain more darkly than regions that replicated in the presence of BrdU. Thus it may be inferred that label was introduced progressively later in the cell cycle moving from a) to d) in figure 4 since a) shows the greatest number of dark bands and d) the fewest.

It may be seen from figure 5 that, as implied in the previous section, the 14^X containing the late replicating X segment can be distinguished from the 14^X containing the early replicating X

Figure 1: A quinacrine-banded karyotype of KOP-2.

Figure 2: A partial metaphase spread photographed after the T pulse. The arrowhead indicates 14^{X}_{L}, and the arrow indicates 14^{X}_{E}. There is a satellite association between the 14^{X}_{E} and a G group chromosome.

Figure 3: A metaphase spread photographed after the B pulse. The arrowhead indicates 14^{X}_{L}, and the arrow indicates 14^{X}_{E}.

Fig. 1

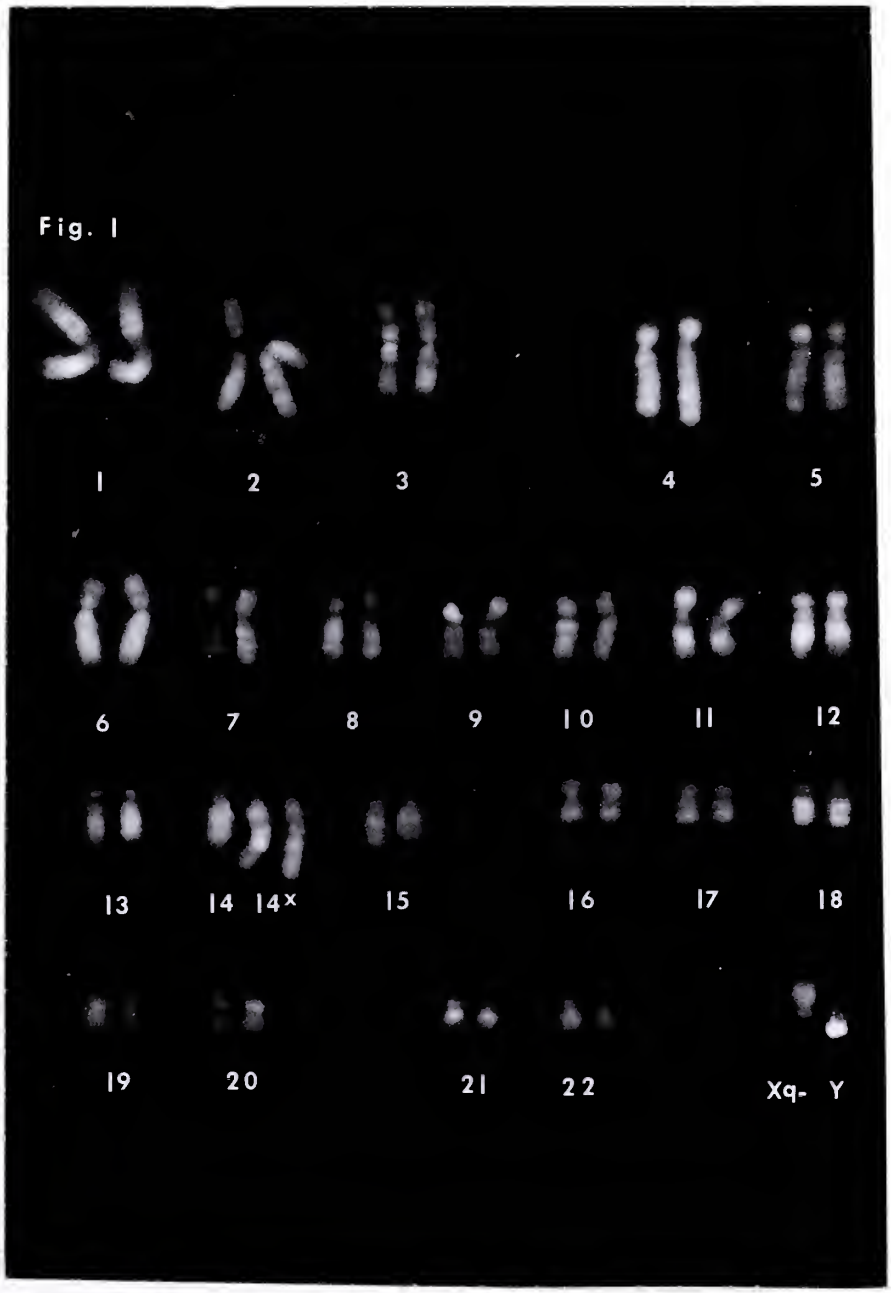


Fig. 2

Fig. 3

segment purely on the basis of the staining of the X segments of these chromosomes. In the $14^{X}E$, the band most predominantly stained was Xq21 while in the $14^{X}L$, the bands most predominantly stained were Xq25 and Xq27. Cell h) demonstrates this well -- arrow 1 indicates Xq21 and arrow 2 indicates Xq25 and Xq27.

The T pulse demonstrated several different patterns of spreading of late replication (figure 5). For reference, a schematic drawing of the bands present on the 14^{X} chromosomes is included with fig. 5. In the following discussion the terms "proximal" and "distal" specify relationship to the X derived material in the 14^{X} chromosomes.

The cell shown in figure 5 a) had no autosomal band in $14^{X}L$ which was darker than the corresponding band in $14^{X}E$ or in 14. In fact 14q31, the band of 14^{X} which is closest to the X derived material (see arrow) is lighter (and by inference earlier replicating) in $14^{X}L$ than in $14^{X}E$ or in 14. This implies that there was no spreading of late replication beyond the X portion of the $14^{X}L$ chromosome in this cell.

The cell shown in figure 5 b) has band 14q31 darker in $14^{X}L$ than in the other two chromosomes (see arrow) but shows equal staining elsewhere. A staining difference in band 14q31 implies that replication is asynchronous in the two 14^{X} chromosomes at the level of 14q31. This suggests that, in $14^{X}L$, spreading of late replication has extended from the X derived portion of the chromosome at least as far as 14q31. However, equal staining of $14^{X}E$ and $14^{X}L$ in 14q23, the quinacrine-bright band just beyond 14q31, implies that spreading has not gone as far

Figure 4: Chromosome # 1's from four T pulse metaphases. Thymidine was introduced progressively later in the cell cycle moving from a) to d).

Figure 5: Eight T pulse partial karyotypes exhibiting a variety of spreading patterns. Chromosomes 1, 14, $14^{X}E$, and $14^{X}L$ are shown. Each unlabeled arrowhead indicates the furthest locus from the X segment of the $14^{X}L$ which is darker in $14^{X}L$ than in $14^{X}E$. Labeled arrowheads indicate the bands of the X segment of the 14^{X} which distinguish $14^{X}E$ from $14^{X}L$. A schematic drawing of the bands present on the 14^{X} chromosomes appears on the page preceding figure 5. In this drawing, Q-bright bands are shaded.

Fig.4

a b c d

Paris Conference Bands in the 14^{X} of KOP-2

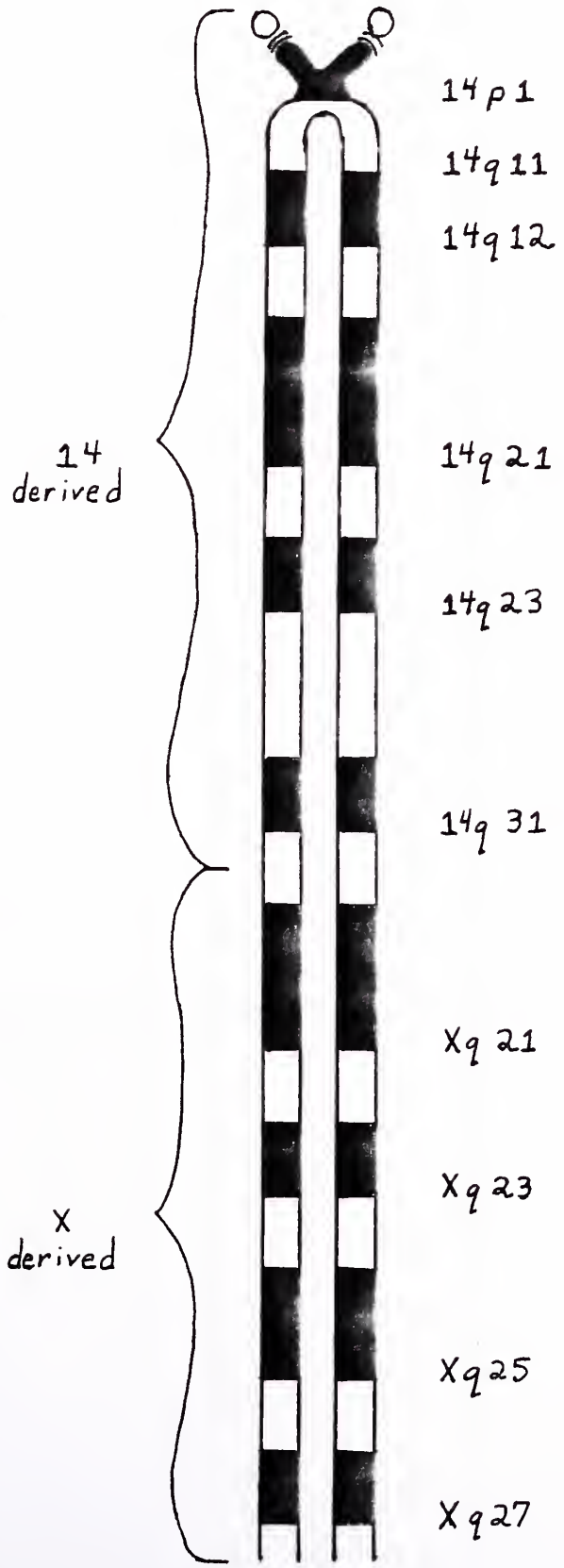
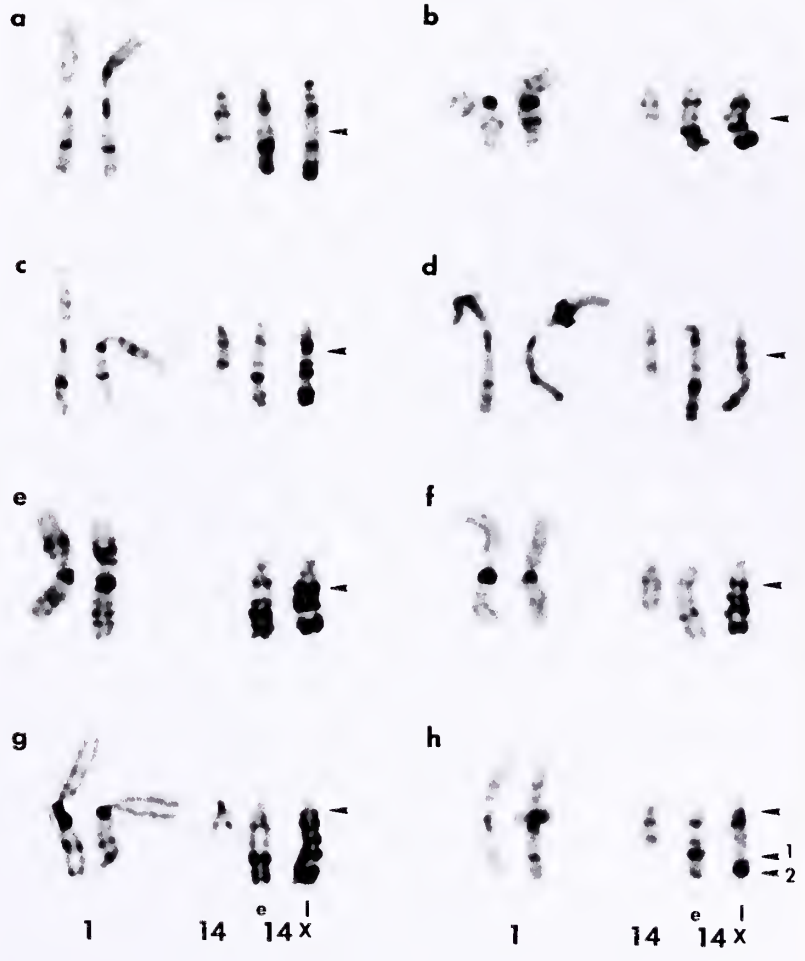


Fig.5



as 14q23. This is true because, as will be shown in the quantitative section below, equal staining of the $14^{X}E$ and the $14^{X}L$ at 14q23 (or at 14q12) implies synchronous replication in $14^{X}E$ and $14^{X}L$ at the level of 14q23 (or of 14q12 respectively). Thus the cell shown in 5 b) exhibits termination of the spreading of late replication between the 14q31 and 14q23 bands of its $14^{X}L$ chromosome.

In the cells shown in figure 5 c) and 5 d), 14q31 is somewhat darker in $14^{X}L$ than in $14^{X}E$ or in 14, 14q23 is much darker in $14^{X}L$ than in $14^{X}E$ or in 14 (see arrows), and the other bands have equal staining. In these cells spreading has gone at least as far as band 14q23. The extent beyond 14q23 to which there has been spreading of late replication was deduced as follows. Equal staining of the $14^{X}L$ and the $14^{X}E$ at band 14q21 (the quinacrine-bright band just distal to 14q23) does not necessarily prove that 14q21 in the $14^{X}L$ chromosome replicates at the same time that it does in the $14^{X}E$ chromosome. That is, equal staining at the level of band 14q21 does not necessarily prove that spreading of late replication stops before reaching 14q21. This is so because, in the T pulse, thymidine was given to certain cells early enough in the cell cycle so that band 14q21 on the <u>early</u> replicating 14^{X} was darkly stained (see quantitative section below). In such cells, it is impossible to determine whether 14q21 on the $14^{X}L$ replicated "early" or "late"

since the band would have stained darkly in either case. Thus in such cells, 14q21 will stain equally on all 14-bearing chromosomes whether there is synchronous replication at this level or not. On the other hand, interpretation of the equality of staining intensity in band 14q12 is not subject to this ambiguity: equality here implies lack of spreading (again, see below). Thus spreading of late replication in the cells shown in figure 5 c) and d) terminates between band 14q23 and band 14q12 on $14^{X}L$.

The cells shown in figure 5 e) and f) have a darker band 14q31, 14q23, and 14q21 (see arrows) on $14^{X}L$ than they do on $14^{X}E$ or 14. The disparity in staining intensity in these regions indicates that there is spreading of late replication on the $14^{X}L$ at least as far as 14q21 in these cells. The equality of stain intensity at 14q12 implies (as was noted) a lack of spreading to this region. Thus cells e) and f) of figure 5 show termination of spreading of late replication between band 14q21 and band 14q12 of the $14^{X}L$ chromosome.

Cells g) and h) of figure 5 show darker staining in the 14q31, 14q23, 14q21 and 14q12 bands of the $14^{X}L$ (see unlabeled arrows) than of the $14^{X}E$ or of the 14. They therefore exhibit spreading on $14^{X}L$ beyond the level of band 14q 12.

It may be seen from figure 5 that the extent of spreading in a given cell was independent of the time at which the cell was

labeled as determined by the number of bands present on chromosome #1. For example, the cells shown in 5 e) and f) have the same degree of spreading, but show markedly different labeling times on the basis of their chromosome 1's while the cells shown in b) and h) have a very similar staining of their #1's but have quite different spreading patterns.

An attempt was made to quantitate the above observations by determining the relative number of cells exhibiting each type of spreading pattern. The staining intensities of bands 14q31, 14q23, 14q21, 14q12, and 14p1 on the 14^Xs and on the normal 14 were assigned numerical values using the scale 0-3 as previously described. For each band, the assignments of stain intensity made visually were used to construct plots of the difference in intensity between that band on the 14^X_E and on the 14 (i.e. "14^X_E-14") versus the duration of the chromosomes' exposure to thymidine. Similarly plots of "14^X_L-14" and of "14^X_L-14^X_E" versus the duration of exposure to thymidine were made. These graphs are presented in figures 6 a-c, 7 a-c, 8 a-c, 9 a-c, and 10 a-c. In addition, for each band on each of the three chromosomes plots were made of the average staining intensity over each 1/4 of the series based upon the duration of exposure to thymidine -- the average stain intensity for a given band on a given chromosome in cells #1-#9

in the series was compared to the average in cells #10-#18, etc. These plots are shown in figures 6d, 7d, 8d, 9d, and 10d. The test of statistical significance employed was the t test for the difference of means; the level of significance chosen was $p \leq .025$. Critical values for each of the graphs described above appear in Table 1.

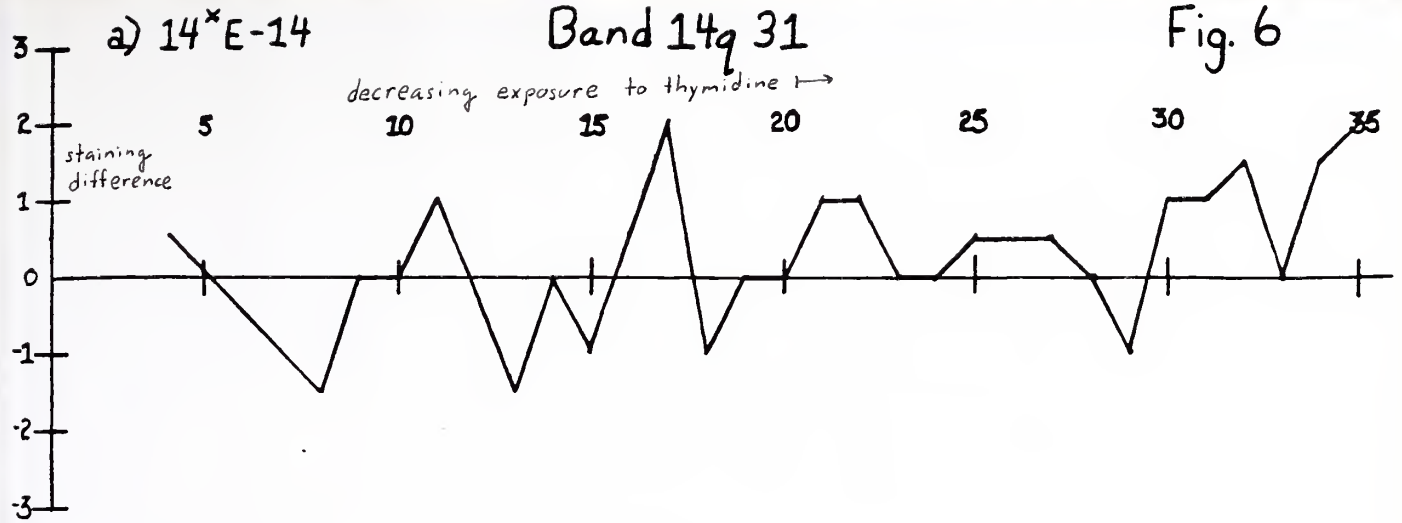
Plots of the difference between the staining of the $14^{X}E$ and the 14 for each autosomal band showed that there was no significant difference between staining intensities on these two chromosomes irrespective of the point in the cell cycle at which label was introduced. Comparisons of the mean staining intensities of the $14^{X}E$ with those of the 14 gave the same result. On the other hand, the "$14^{X}L$-14" and "$14^{X}L$-$14^{X}E$" graphs and the plots of mean staining intensity showed that there were differences between the staining intensity of the <u>late</u> replicating 14^{X} and the other two chromosomes.

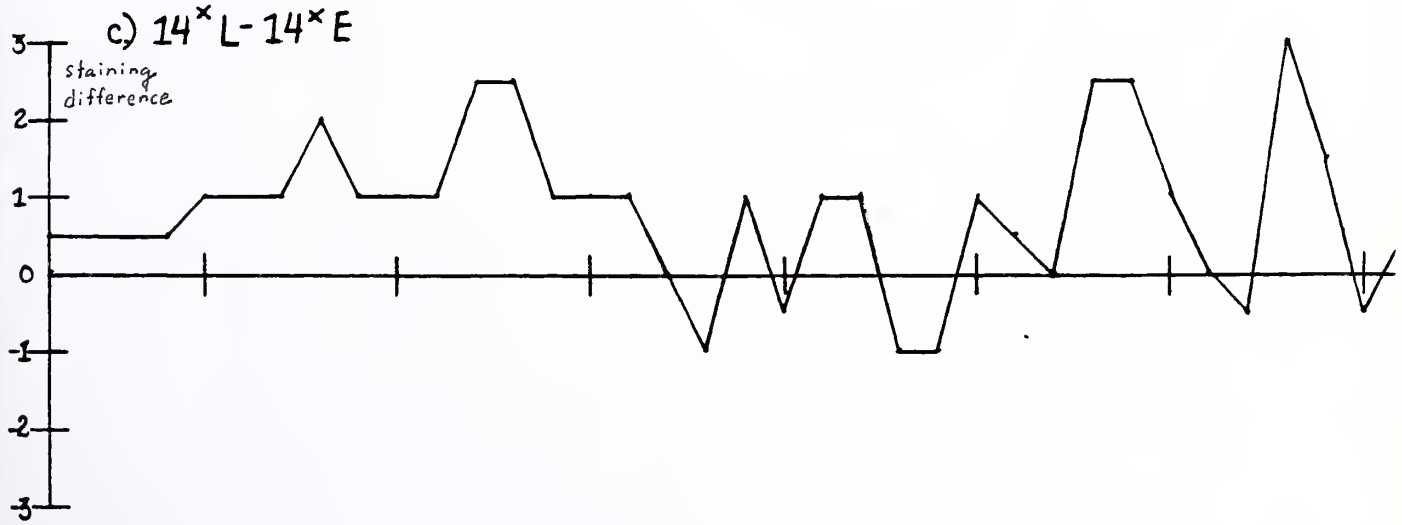
Band 14q31 was significantly darker in the $14^{X}L$ chromosome than in the $14^{X}E$ or in the 14 in cells from all portions of the labeling series. This conclusion is based upon the results shown by both graphing methods. In general, the band was morphologically identifiable on all three chromosomes but tended to be significantly darker on the $14^{X}L$ than on the $14^{X}E$ or the 14.

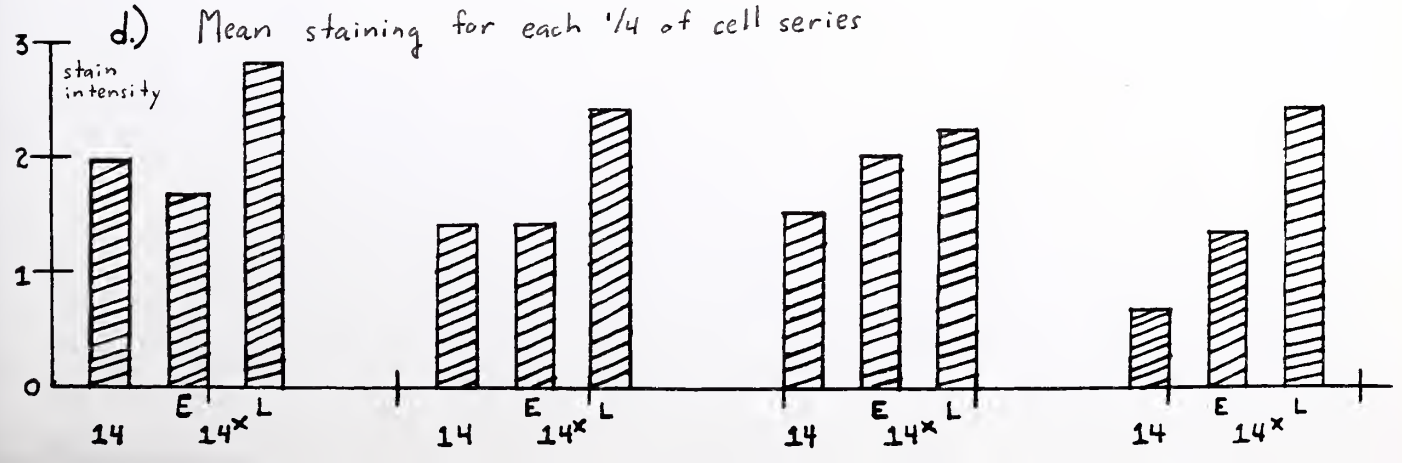
Figures 6-10: Staining intensity of five bands on the 14, $14^{X}E$, and $14^{X}L$ in the T pulse. Figure 6 pertains to band 14q31, figure 7 to 14q23, figure 8 to 14q21, figure 9 to 14q12, and figure 10 to 14p1. In each figure part a) shows the series based on the duration of exposure to thymidine (cell #1 having the longest exposure and cell #36 the shortest) versus the staining difference between $14^{X}E$ and 14 ("$14^{X}E$-14"). Part b) shows this series of cells versus "$14^{X}L$-14". Part c) shows this series versus "$14^{X}L$-$14^{X}E$". Part d) shows the mean staining intensity on 14, $14^{X}E$, and $14^{X}L$ for each 1/4 of the cell series.

Band 14q 31

Fig. 6

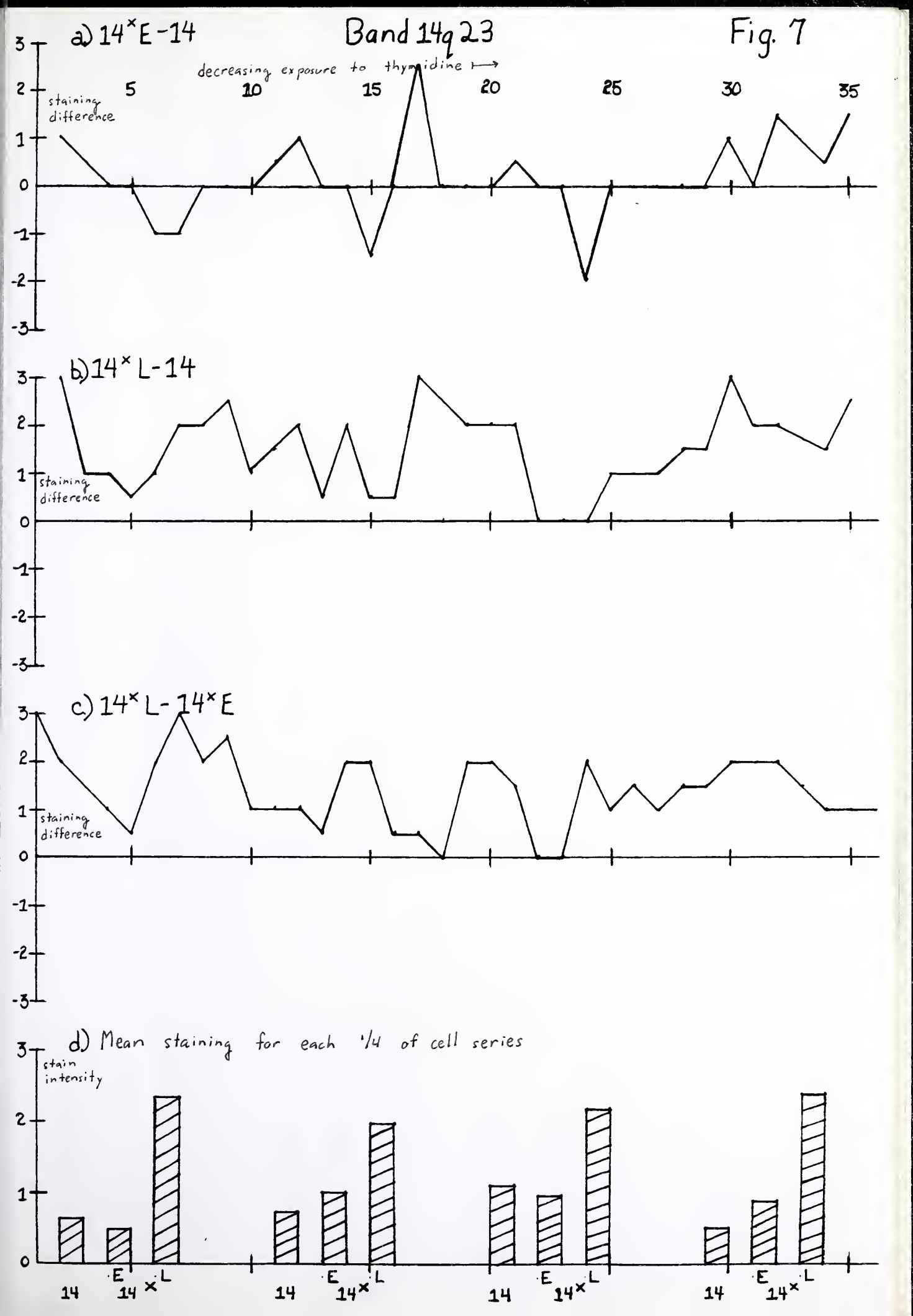






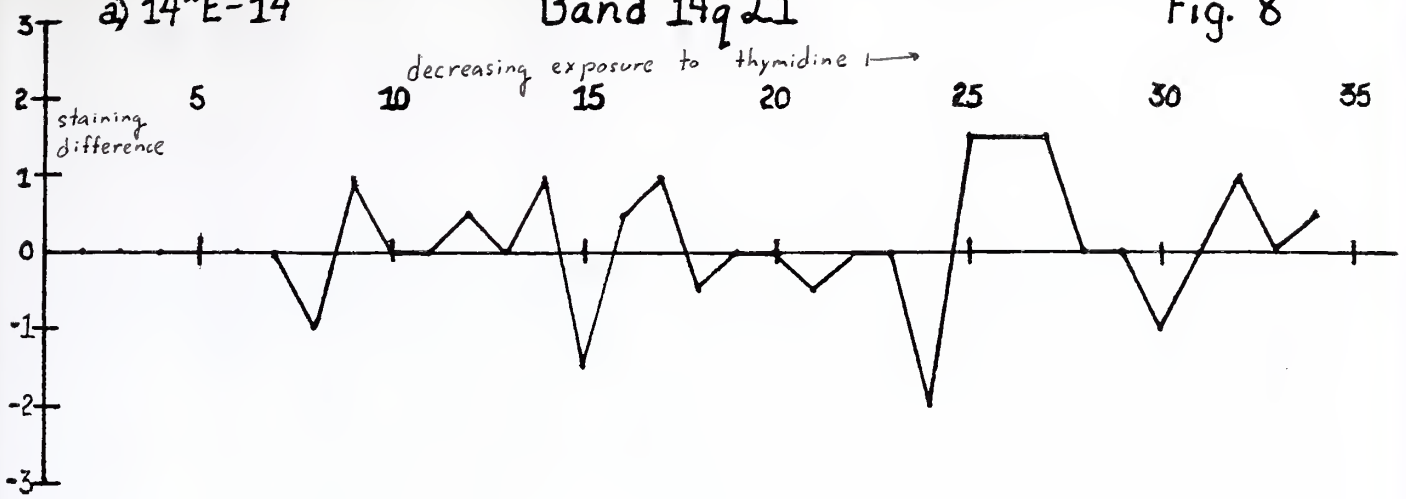
Band 14q23

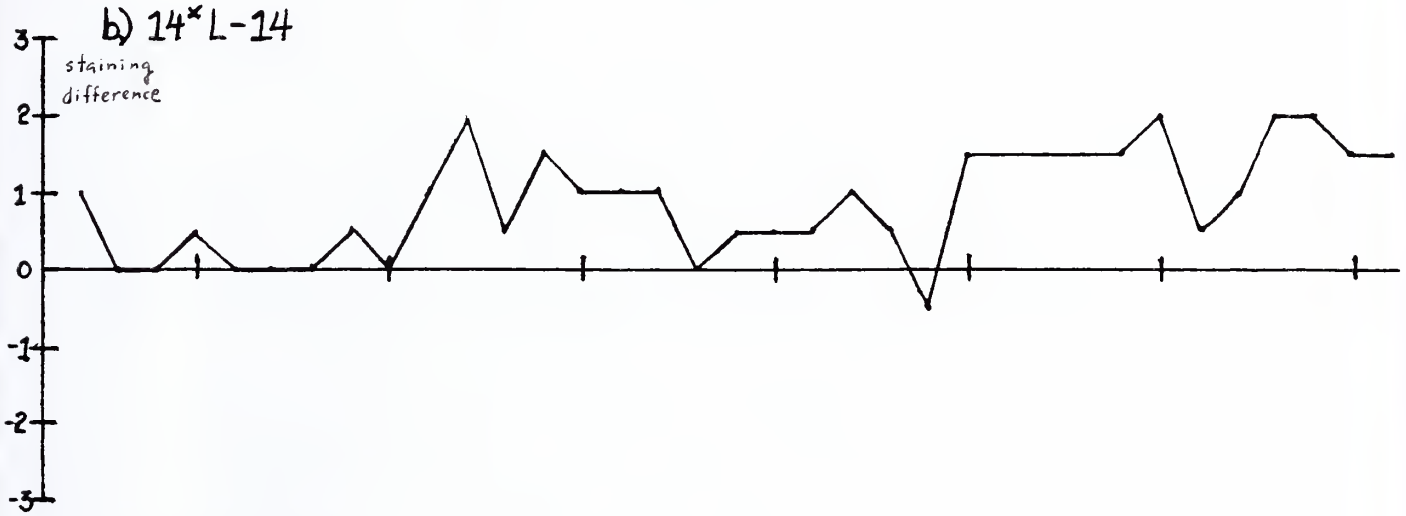
Fig. 7

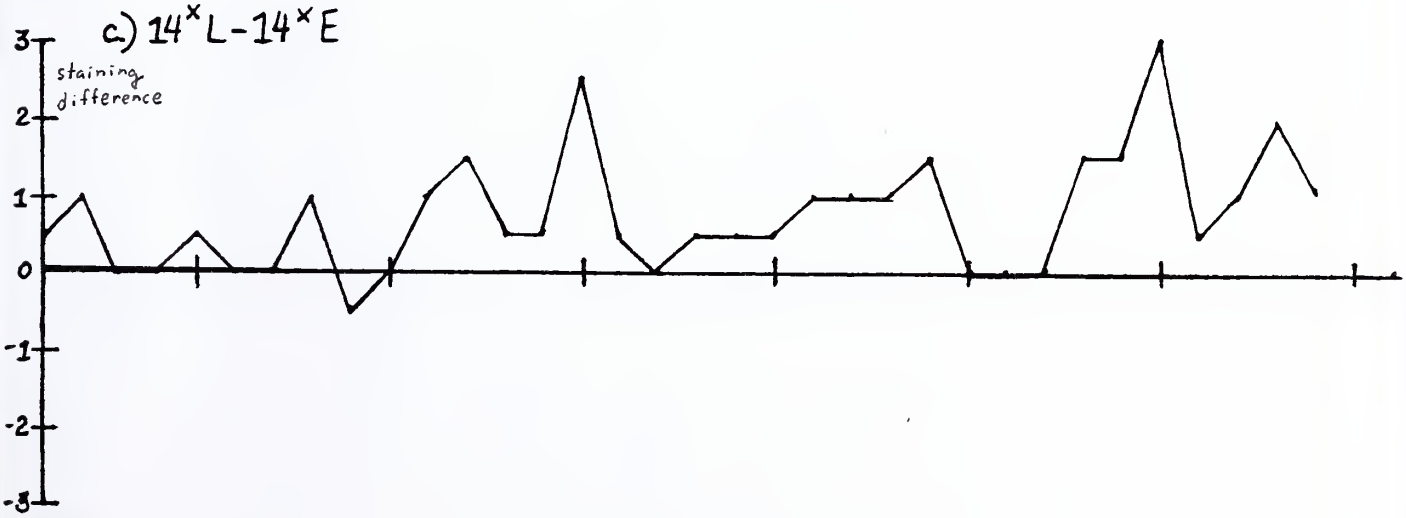


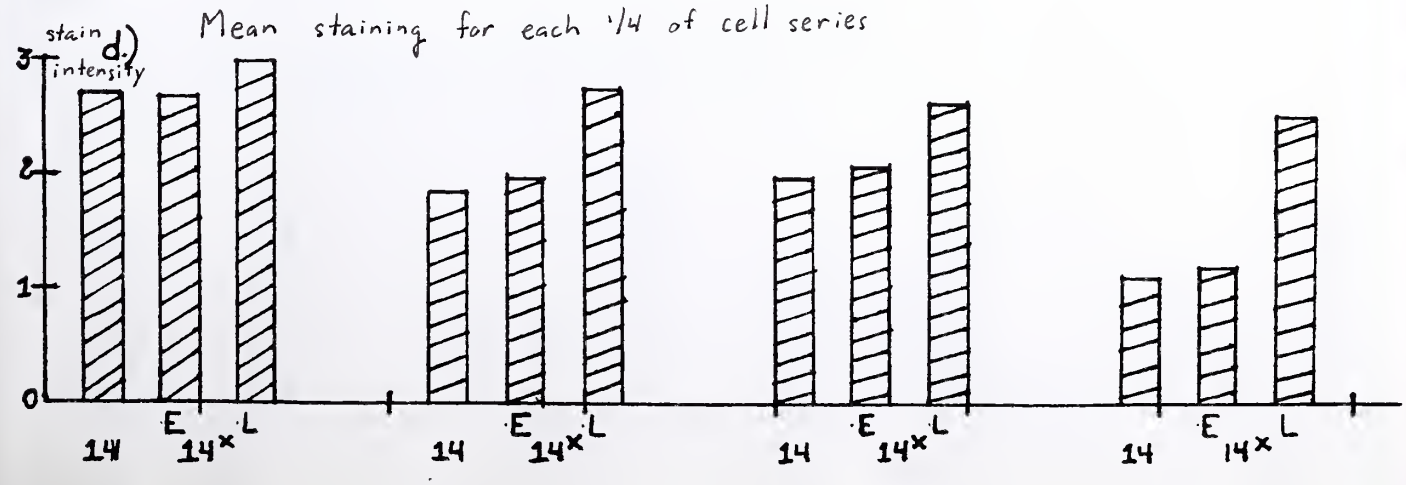
Band 14q21

Fig. 8



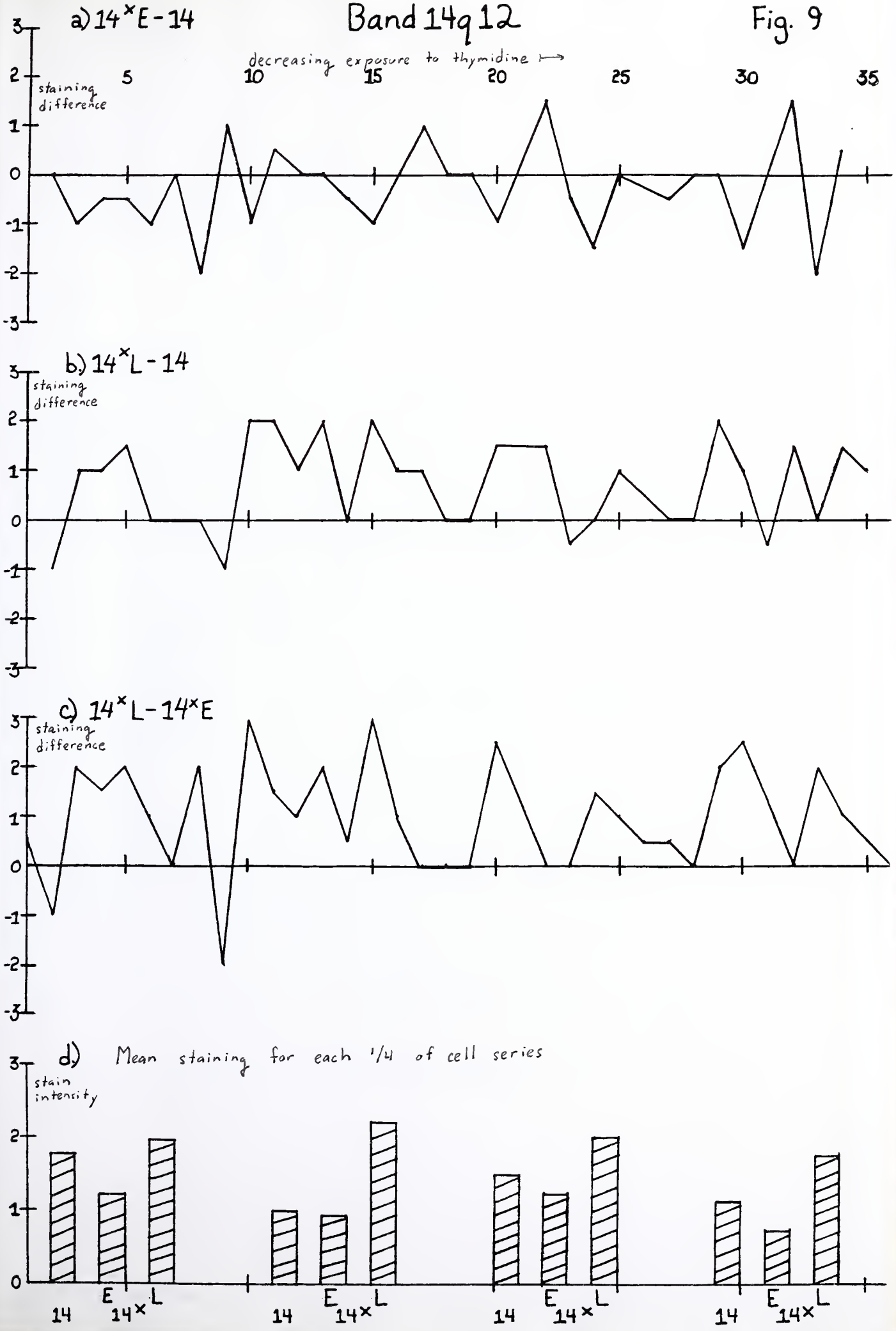






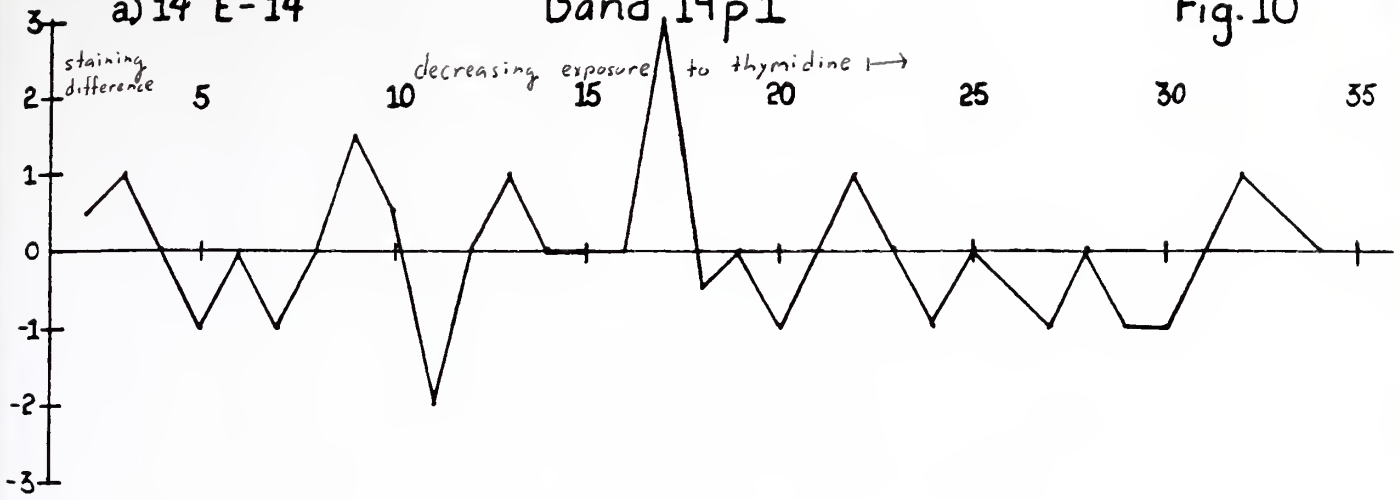
Band 14q12

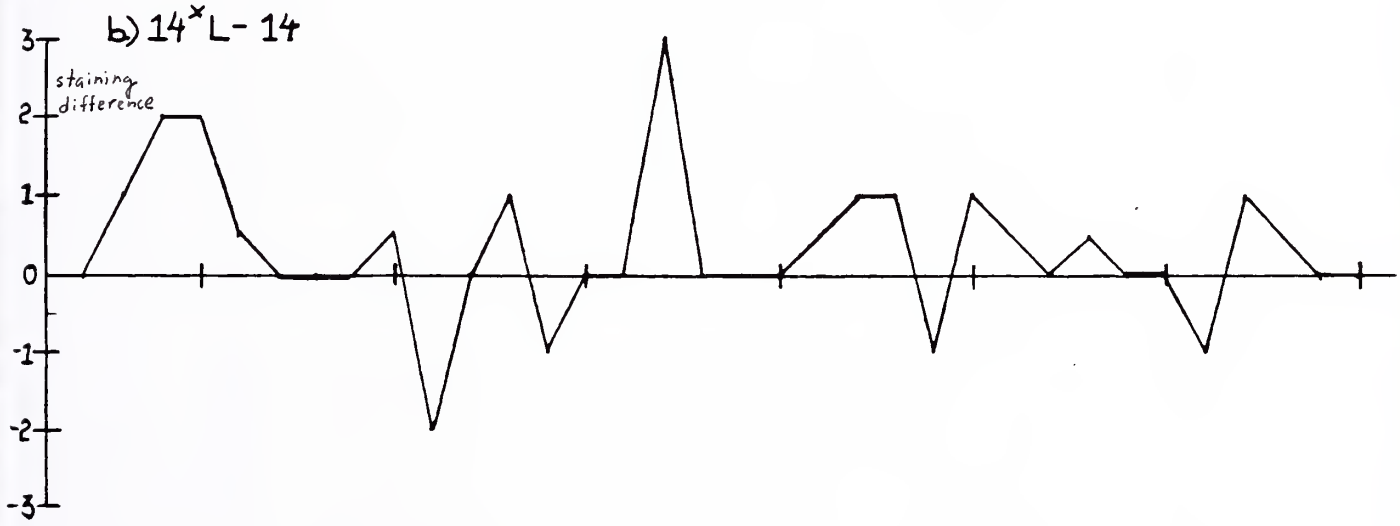
Fig. 9

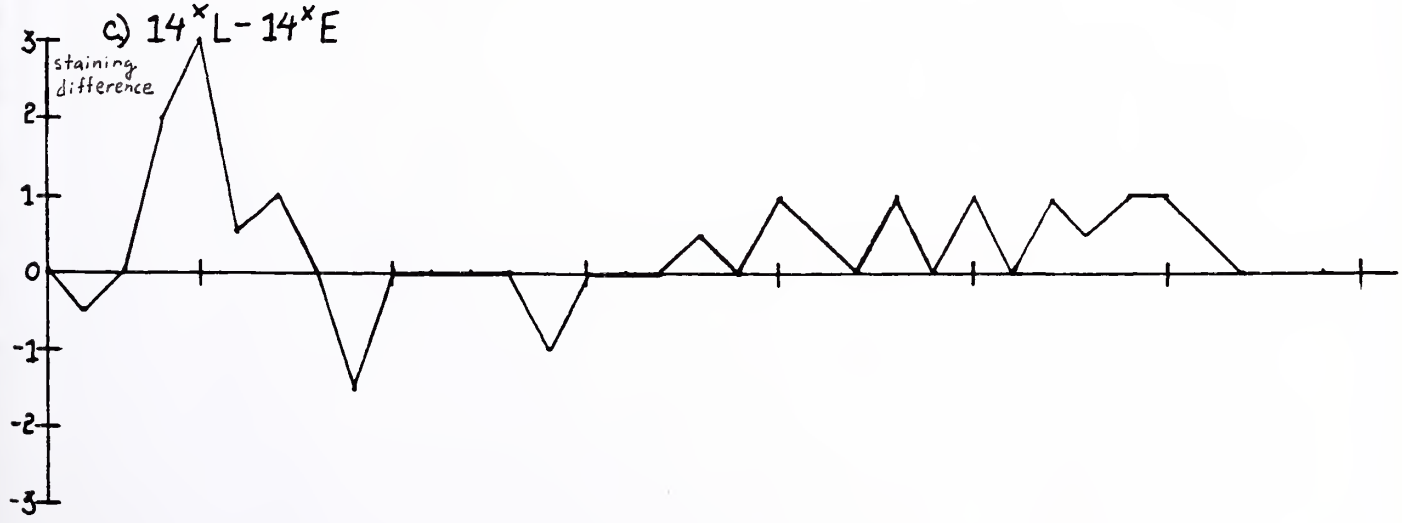


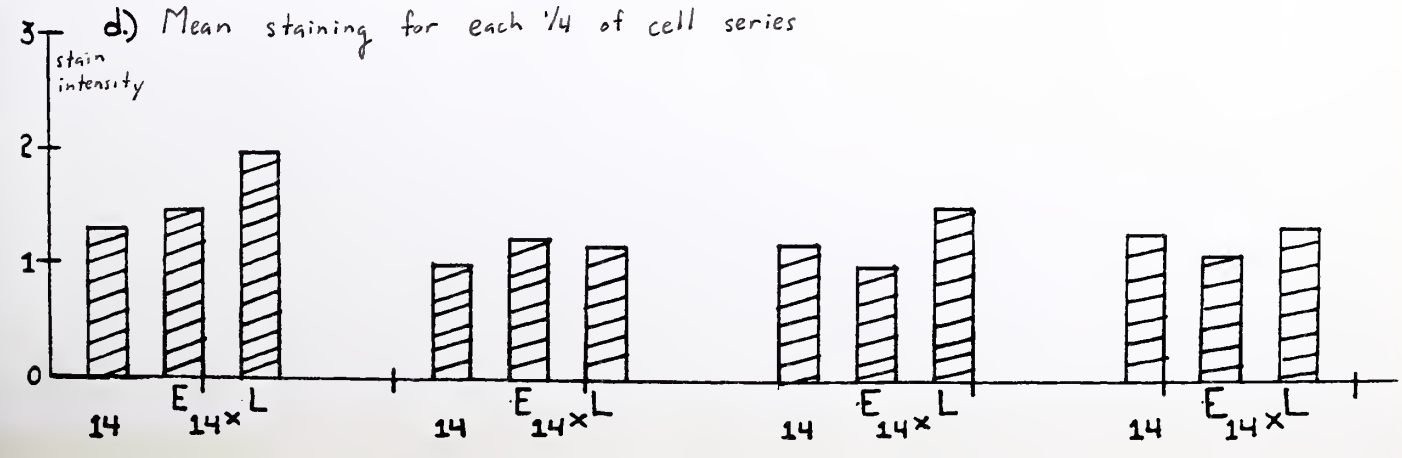
Band 14p1

Fig. 10









14q23 was the band in which the staining difference between the $14^{X}L$ chromosome and the $14^{X}E$ and 14 chromosomes had the greatest magnitude. Both graphing methods indicated that 14q23 was stained most darkly in the $14^{X}L$ in all portions of the labeling series. The band tended to be quite dark on the $14^{X}L$ chromosome but pale on the other two chromosomes.

Band 14q21 stained relatively darkly on all $14^{X}L$, $14^{X}E$, and 14 chromosomes examined. On the basis of both graphing methods, the band stained approximately equally on all three chromosomes in the first 1/4 of the labeling series (cells #1-#9). However, the band was significantly darker on the $14^{X}L$ than on the $14^{X}E$ or the 14 for the last 3/4 of the labeling series (cells #10-#36).

14q12 is the Q-bright band on 14q which is closest to the centromere. 14q12 was darkest on the $14^{X}L$ chromosome with the exception of the cells in the first 1/4 of the labeling series; in these cells it had approximately equal intensity in the normal 14 $14^{X}E$, and $14^{X}L$. Band 14q12 was almost always darkly stained on the $14^{X}L$ chromosome but was as pale as the surrounding "early replicating" quinacrine-dull bands in a significant number of the $14^{X}E$ and 14 chromosomes.

Band 14p1, the band most distal to the translocated X material, was technically difficult to score for staining intensity because

of the condensation of the DNA at the level of the chromosomes' secondary constrictions. The subdivisions of 14p1: 14p11, 14p12, and 14p13, were not distinguishable, as such, on any chromosome. There was no significant difference between the staining intensity of 14p1 on chromosomes $14^{X}L$, $14^{X}E$, and 14.

In order to determine the degree of spreading of late replication in each late replicating 14^{X} chromosome, the above data were organized in the following way. For each of the 36 cells studied, a diagram of the cell's $14^{X}L$ chromosome was made (figure 12) showing each of the described autosomal bands. Each band which was at least one scoring unit darker on the $14^{X}L$ chromosome than on the $14^{X}E$ was marked. The staining intensity of the $14^{X}L$ was compared with that of the $14^{X}E$ but not the 14 in order to avoid dealing with the problem of "normal" replication asynchrony between homologues that would be introduced in a comparison of the $14^{X}L$ with the 14. The tolerance of one scoring unit was arrived upon because it corresponded, on our photographs, to the minimal scoring difference between "present" and "absent" bands -- this distinction being the usual criterion for replication asynchrony in terminal labeling techniques involving BrdU.

On the basis of the number and location of bands showing unequal staining on the $14^{X}L$ and the $14^{X}E$ chromosomes, the

Table 1 and Table 3: These tables show the t test for the difference of means between the 14, $14^{X}E$, and $14^{X}L$ (pairwise) for each autosomal band. s_p is an estimator of the pooled population variance of the bands being compared. H_0 is the null hypothesis. The 95% confidence interval is calculated as follows:

$$\mu_1 - \mu_2 = (\bar{X}_1 - \bar{X}_2) \pm t_{.025}\, s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where $$s^2_p = \frac{1}{n_1 + n_2 - 2} \left[\sum_{i=1}^{n_1} (X_{1i} - \bar{X}_1) + \sum_{i=1}^{n_2} (X_{2i} - \bar{X}_2) \right]$$

μ = population mean

$\bar{X}$ = sample mean

n = sample number

t = student's t with $n_1 + n_2 - 2$ d.f.

TABLE 1

Band	Mean difference	s_p	95% confidence interval	accept H_o?
14q31				
a) 14^X_E-14	0.22	0.75	-.15 to .59	Yes
b) 14^X_L-14	1.07	0.74	.71 to 1.43	No
c) 14^X_L-14^X_E	0.85	0.76	.48 to 1.22	No
14q23				
a) 14^X_E-14	0.08	0.71	-.26 to .42	Yes
b) 14^X_L-14	1.46	0.76	1.09 to 1.83	No
c) 14^X_L-14^X_E	1.38	0.78	1.01 to 1.75	No
14q21				
a) 14^X_E-14				
first 1/4	.02	0.44	-.44 to .48	Yes
last 3/4	.12	0.71	-.28 to .62	Yes
b) 14^X_L-14				
first 1/4	.25	0.34	-.1 to .60	Yes
last 3/4	.98	0.62	.64 to 1.32	No
c) 14^X_L-14^X_E				
first 1/4	.27	0.33	-.06 to .60	Yes
last 3/4	.86	0.75	.45 to 1.27	No

see over

TABLE 1 (Continued)

Band	Mean difference	s_p	95% confidence interval	accept H_o?
14q12				
a) 14^X_E-14				
first 1/4	-.53	1.02	-1.59 to .53	Yes
last 3/4	-.26	0.85	-1.12 to .60	Yes
b) 14^X_L-14				
first 1/4	.14	1.05	-.94 to 1.22	Yes
last 3/4	.77	0.72	.36 to 1.18	No
c) 14^X_L-14^X_E				
first 1/4	.67	1.12	-.44 to 1.78	Yes
last 3/4	1.03	0.88	.53 to 1.53	No
14p1				
a) 14^X_E-14	-.01	0.85	-.44 to .42	Yes
b) 14^X_L-14	.28	0.86	-.15 to .71	Yes
c) 14^X_L-14^X_E	.29	0.84	-.12 to .70	Yes

location on $14^{X}L$ at which termination of spreading of late replication occurred was determined. The following rules were used in making this determination:

1) A model of spreading in which there is no "skipping" of large chromosomal regions (regions as large as chromosomal bands) is assumed.

2) A $14^{X}L$ chromosome stained at least one staining unit more darkly than the corresponding $14^{X}E$ chromosome at the level of a given band is said to have spreading of late replication up to and including this band.

3) The extent of spreading in a $14^{X}L$ chromosome which has one or more bands with equal staining intensity on the $14^{X}L$ and the $14^{X}E$ is posited as follows:

a) if band 14q12 is equally stained in $14^{X}L$ and in $14^{X}E$, or if band 14q23 is equally stained in $14^{X}L$ and in $14^{X}E$, then spreading of late replication has terminated before reaching 14q12 or 14q23 respectively.

b) if band 14q21 is equally stained in $14^{X}L$ and in $14^{X}E$, or if band 14q31 is equally stained in $14^{X}L$ and in $14^{X}E$, no conclusion can be made about whether spreading has extended into 14q21 or into 14q31 respectively.

(Justification for rule #3: Rule 3 was arrived upon after a careful analysis of when during the cell cycle the cells under

study were exposed to thymidine label. When two chromosomal bands stain equally in the T Pulse protocol, it must be determined whether the equal staining indicates synchronous replication of the two bands or whether it merely reflects addition of label before both bands begin replication or after both bands finish replication. For our cells, this determination was made by making the following observations.

Consider figure 11. Figure 11 shows the approximate replication times for bands 14q12, 14q21, 14q23, and 14q31 based on previously published data (66). The black horizontal lines indicate replication times for the early 14^X while the red horizontal lines indicate replication times for the late 14^X assuming spreading of late replication through all four bands. The late replicating bands of the $14^X L$ are pictured as replicating in a pattern which is very similar to that of the corresponding bands of the $14^X E$ but this is not essential for the arguments that follow and indeed may not be true. Overlaps of the "early" and "late" replication times of a given band are possible but the existence of such overlaps would not change the qualitative conclusions presented below either. The vertical lines marked 1, 2, 3, and 4 indicate hypothetical labeling times. Once label is introduced, it remains present throughout the rest of the cells' DNA synthetic period.

Figure 11: A diagram of early and late replication times for four bands on the autosomal segment of 14^X. The abscissa is time. The horizontal black lines indicate the end of replication in bands when they are early replicating. The horizontal red lines indicate the beginning of replication in bands when they are late replicating. The numbered vertical black lines indicate hypothetical labeling times. Once label is introduced, it is present throughout the rest of the cell's DNA synthetic period.

Figure 12: The extent to which staining difference between $14^X E$ and $14^X L$ has spread based upon the T pulse data. 36 $14^X L$ chromosomes are diagrammed in order of decreasing exposure to thymidine label. Boxes containing parallel diagonal lines indicate bands which were at least 1 staining unit darker on $14^X L$ than on $14^X E$. Single diagonal slashes indicate bands which were expected to stain 1 unit darker on $14^X L$ than on $14^X E$ based upon the staining differences at other bands but which were actually only .5 units darker on $14^X L$ than on $14^X E$. ?'s indicate bands in which a comparison of $14^X E$ and $14^X L$ was not possible. Vertical red lines show the chromosomal segments in which spreading of late replication must have terminated.

Fig 11

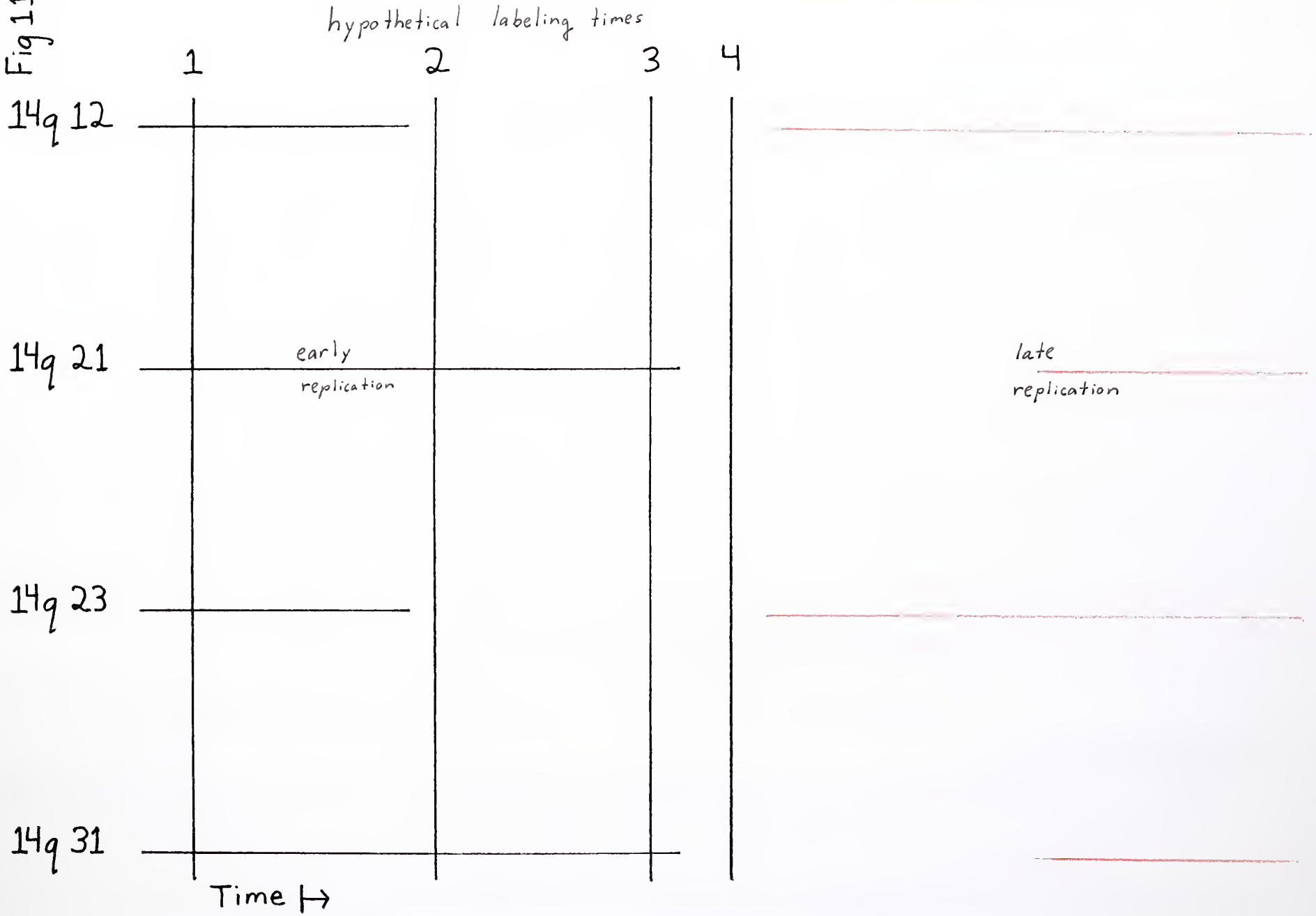
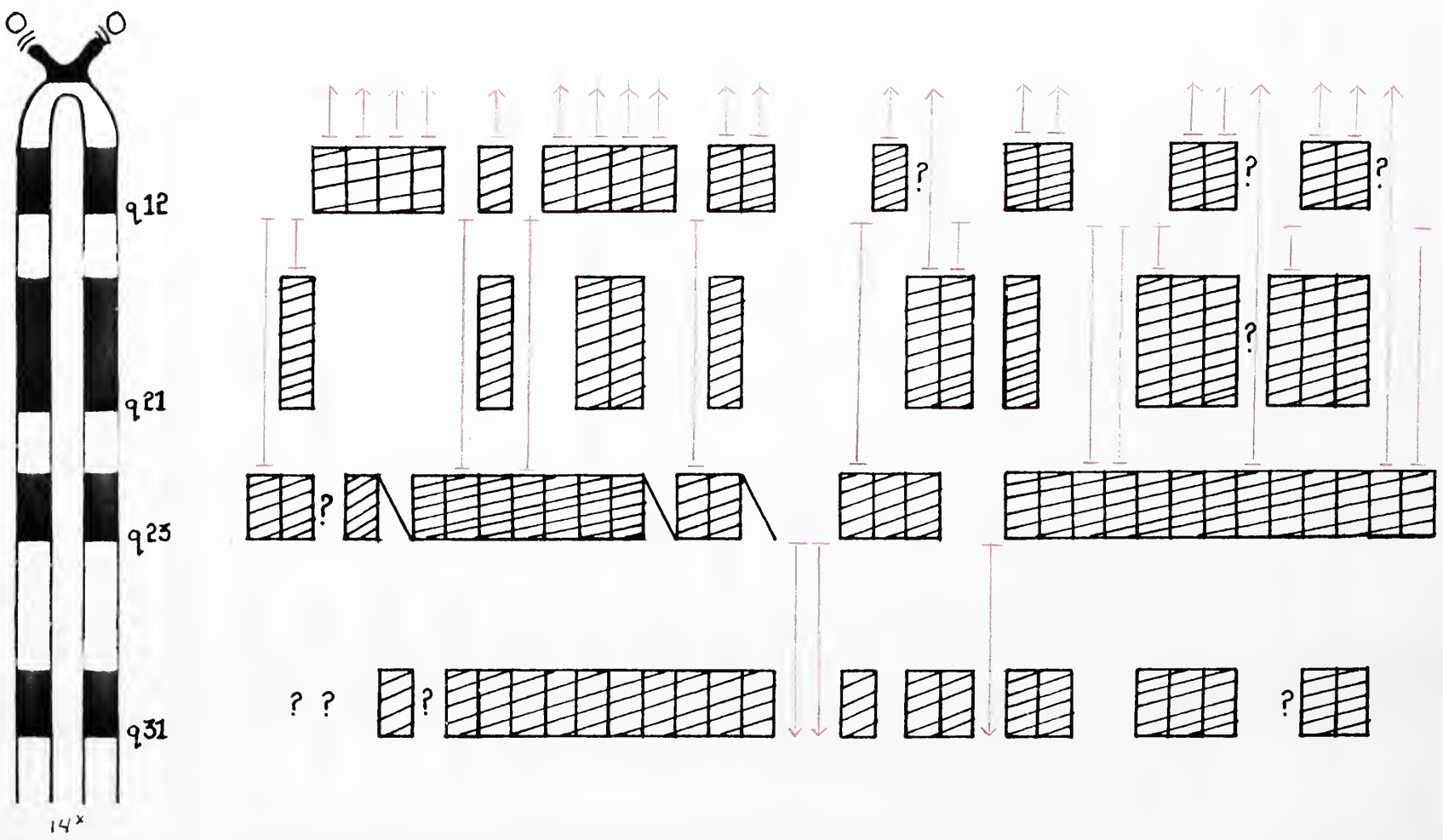

hypothetical labeling times
1
2
3
4
14q 12
14q 21
early replication
14q 23
14q 31
late replication
Time ↦

Fig. 12


cells in order of decreasing exposure to thymidine
1
5
10
15
20
25
30
35
q12
q21
q23
q31
?

14x

If any of the cells studied were labeled at time 1, all the bands of the $14^{X}E$ would stain fairly darkly as would all the bands of the $14^{X}L$ whether they are late replicating or not. This would mean that, in these cells, equality of staining intensity would not necessarily imply synchronous replication for any autosomal band. No cells in our study did stain at time 1 however, because no $14^{X}E$ chromosome displayed a dark band 14q23.

If any cells studied were labeled at or around time 2, the $14^{X}E$ would have bands 14q12 and 14q23 remaining light while bands 14q21 and 14q31 would stain dark. The $14^{X}L$ would have 14q21 and 14q31 dark if these bands had replicated early or if they had replicated late, but 14q12 and 14q23 would be dark only if they had replicated late. Thus, for this staining time, equality of staining intensity implies synchronous replication only for bands 14q12 and 14q23. In our study, certain cells did stain at time 2. This is known because in some cells $14^{X}E$ had bands 14q21 and 14q31 darkly stained and bands 14q12 and 14q23 palely stained. It is also known because several cells showed a staining difference between $14^{X}E$ and $14^{X}L$ at band 14q12 or at band 14q23 but no difference at a band 14q21 or a band 14q31 which is located closer to the X portion

of the chromosome than is the aforementioned 14q12 or 14q23. By virtue of its interposition between late replicating regions, the band 14q21 or band 14q31 must have been late replicating on $14^{X}L$; the absence of a difference in staining between the two 14^{X}s at this region, then, must reflect a labeling at time 2. (The converse situation -- i.e. the existence of a band 14q12 or band 14q23 with equal staining on $14^{X}E$ and $14^{X}L$ which is proximal to a band 14q21 or band 14q31 showing unequal staining on $14^{X}E$ and $14^{X}L$ -- never occurred.)

If any cells studied were labeled at or around time 3, all four bands in $14^{X}L$ in these cells would have been lighter if they had replicated early than if they had replicated late. Bands 14q21 and 14q31 on $14^{X}E$ and on 14 have not quite finished replicating at time 3, so that they will have a dark "tinge" on these early replicating chromosomes -- however, late replication in these bands should still make them much darker than should early replication. At this labeling time, then, equal staining intensity in $14^{X}E$ and $14^{X}L$ implies synchronous replication for all autosomal bands. Several cells in our study did replicate at time 3. This is true since some cells had $14^{X}E$'s which showed a "dark tinge" of stain in bands 14q21 and 14q31 with light 14q12 and 14q23 and which showed

staining differences between $14^{X}E$ and $14^{X}L$ at the level of all four bands.

No cells in the study were stained during time 4 or later since no $14^{X}E$ chromosome showed a palely stained band 14q 21.

In summary, since cells in our study were labeled only at labeling times near #s 2 and 3 and since it was often difficult to know during which of the two times a particular cell was labeled, rule 3) is appropriate for determining the significance of equal staining of an autosomal band in the $14^{X}E$ and $14^{X}L$.)

Band 14p1 was excluded from the analysis of spreading because the scoring of staining intensity in this region was technically so unsatisfactory.

The bands within which spreading terminated are indicated in red in figure 12. Table 2 gives a profile of the spreading patterns. Discounting indeterminate cells, spreading extended to band 14q31 in 100% of cells, to 14q23 in 83%, to 14q21 in 77%, and to 14q12 in 48%. 95% confidence intervals for these percentages for n = 36 (67) are 14q31: 91-100%, 14q23: 78-97%, 14q21: 75-96%, and 14q12: 37-70%.

TABLE 2: Spreading of late replication in T pulse

Band	# of cells with band inactive	# of cells with band active	# of indeterminate cells	% late replicating	% neglecting indeterminate cells
14q31	33	0	3	92-100	100
14q23	33	3	0	92	92
14q21	23	3	10	64-92	88
14q12	18	15	3	50-58	55

B Pulse:

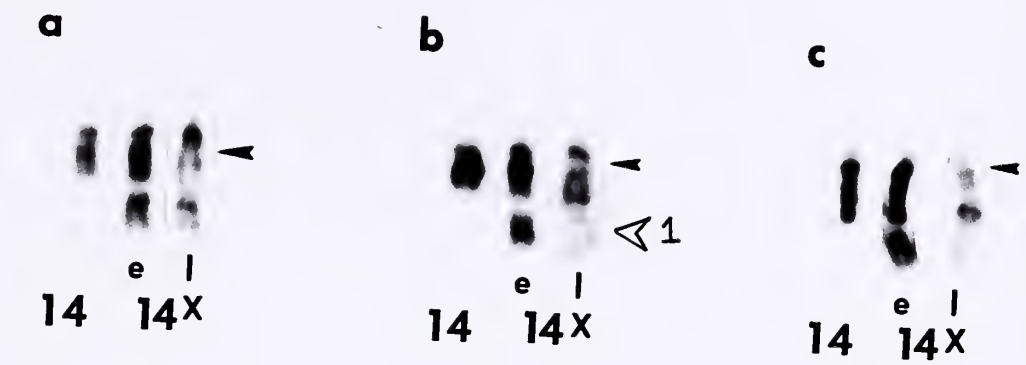
An example of a metaphase photographed as part of the B pulse protocol is shown in figure 3. In this protocol, BrdU was added as a terminal label and late replicating regions stained palely. Early replicating regions (which replicated in the presence of thymidine) were darkly staining. An examination of the staining patterns of the Y and the autosomes produced by the B Pulse revealed no reliable marker for the phase of the cell cycle during which label was introduced; cells were analyzed, therefore, without reference to this variable. In general, analysis of banding intensities in the B Pulse photographs was less satisfactory than in the T Pulse because of the condensation of the chromosomes of cells grown in the thymidine medium.

As in the T pulse, the "late replicating" 14^X was distinguished from the "early replicating" 14^X on the basis of the X portion of the chromosome only. In the B Pulse, the entire Xq22-Xq28 often stained palely in the 14^X chromosome (see labeled arrow, fig. 13 b)).

The B pulse demonstrated several different patterns of spreading of late replication. This is demonstrated by figure 13. The cell shown in figure 13 a) shows the 14^X_L paler than the 14^X_E or the 14 in bands 14q31 and 14q23 (see arrow) but not in bands 14q21, 14q12, 14q11, or 14p1. As will be discussed below, the

Figure 13: Three B pulse partial karyotypes exhibiting several spreading patterns. Chromosomes 14, $14^{X}E$, and $14^{X}L$ are shown. Each unlabeled arrowhead indicates the furthest locus from the X segment of the $14^{X}L$ which is paler in $14^{X}L$ than in $14^{X}E$. The arrowhead labeled "1" indicates the X segment of $14^{X}L$. This segment is often completely pale in the B pulse.

Fig.13



same rules that were used to determine the extent of spreading of late replication in the T pulse are applicable to the B pulse. This being so, a staining difference in bands 14q31 and 14q23 indicates spreading to at least the level of 14q23. An equal staining of the $14^{X}L$, $14^{X}E$, and 14 at band 14q21 does not give information about whether spreading has reached 14q21. However, an equal staining of the $14^{X}L$, $14^{X}E$, and 14 at band 14q12 does imply that spreading of late replication on the $14^{X}L$ has not reached 14q12. Thus, in the cell shown in 13 a), spreading has extended to somewhere between bands 14q23 and 14q12.

The cell shown in 13 b) has paler staining on the $14^{X}L$ than on the $14^{X}E$ or the 14 in bands 14q31, 14q23, and 14q21 (see unlabeled arrow) but not in bands 14q12, 14q11 or 14p1. This implies termination of spreading has occurred between band 14q21 and band 14q12 on the $14^{X}L$ of this cell.

The cell shown in 13 c) has $14^{X}L$ staining more palely than $14^{X}E$ for all autosomal bands. This implies that there is spreading of late replication beyond the level of band 14q11 -- probably to the end of the $14^{X}L$ chromosome in this cell.

A quantitative analysis for determining the relative number of cells exhibiting the various spreading patterns was made. Using a scale of 0-3, the intensities of bands on the $14^{X}L$ were compared with

band intensities on the $14^{X}E$ and the normal 14. For each autosomal band on these chromosomes, the assignments of intensity made visually were used to construct plots of differences in band intensity between the 14-bearing chromosomes versus the number of cells exhibiting each such difference; the differences "$14-14^{X}E$", "$14-14^{X}L$", and "$14^{X}E-14^{X}L$" were used. Mean differences were calculated for each region. These results are shown in figure 14 a-f. The statistical test used was again the t test for the difference of means, $p \leq .025$. Critical values appear in Table 3.

The mean difference between the normal 14 and the $14^{X}E$ was approximately 0 for all bands, with largest standard deviation being 0.18. The distributions were generally symetric around the mean of 0. Thus, there was no significant difference between the staining of the $14^{X}E$ and the 14 in any band.

Band 14q31 had a mean difference in staining intensity of 1.50 between the $14^{X}L$ and the $14^{X}E$ and of 1.44 between the $14^{X}L$ and the 14. These differences are statistically significant. 14q31 on the $14^{X}L$ chromosome was equal in intensity to 14q31 on one of the other two analyzed chromosomes in 4/16 cells and of greater intensity in none.

Band 14q23 had a mean ($14^{X}E-14^{X}L$) of 1.91 and a mean ($14-14^{X}L$) of 1.94. The differences are statistically significant. This

Figure 14: Staining intensity on the 14 and 14^Xs in the B pulse. The empty bars indicate the difference "$14-14^X_E$", the dotted bars indicate "$14-14^X_L$, and the bars containing diagonal lines indicate "$14^X_E-14^X_L$". The number of cells exhibiting staining differences of 2.5 or 3 is compared to the number exhibiting differences of 1.5 or 2, of .5 or 1, of 0, of -1 or -.5, and of -2 or -1.5. The abscissa is the difference in staining intensity between the two indicated chromosomes and the ordinate is the number of cells exhibiting this difference. Vertical arrows indicate the mean differences over all the studied cells. Arrow 1 = mean($14-14^X_E$), arrow 2 = mean($14-14^X_L$), and arrow 3 = mean($14^X_E-14^X_L$). Graph a) is of band 14q31, b) of band 14q23, c) of band 14q21, d) of band 14q12, e) of band 14q11, and f) of band 14p1.

Fig. 14

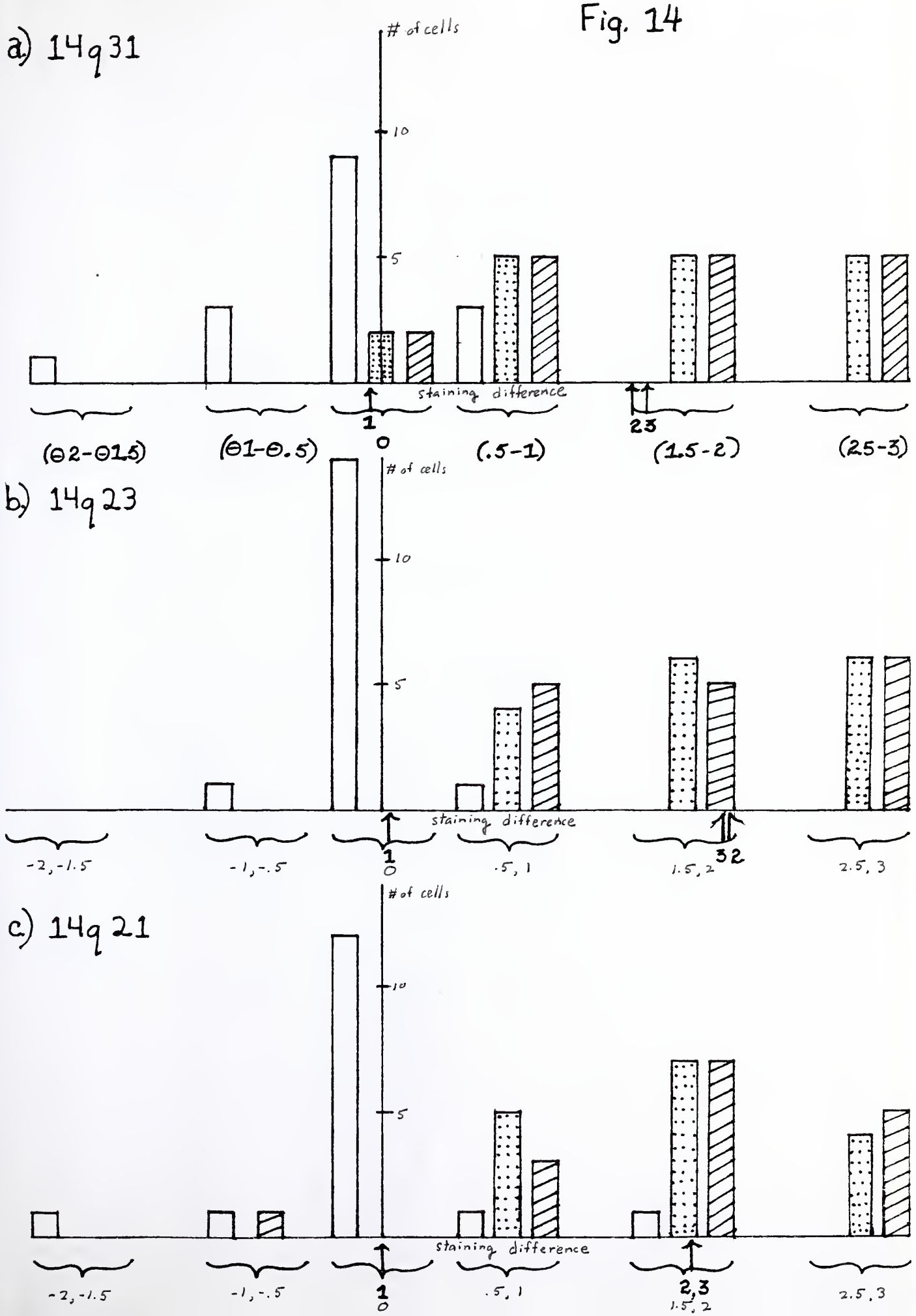
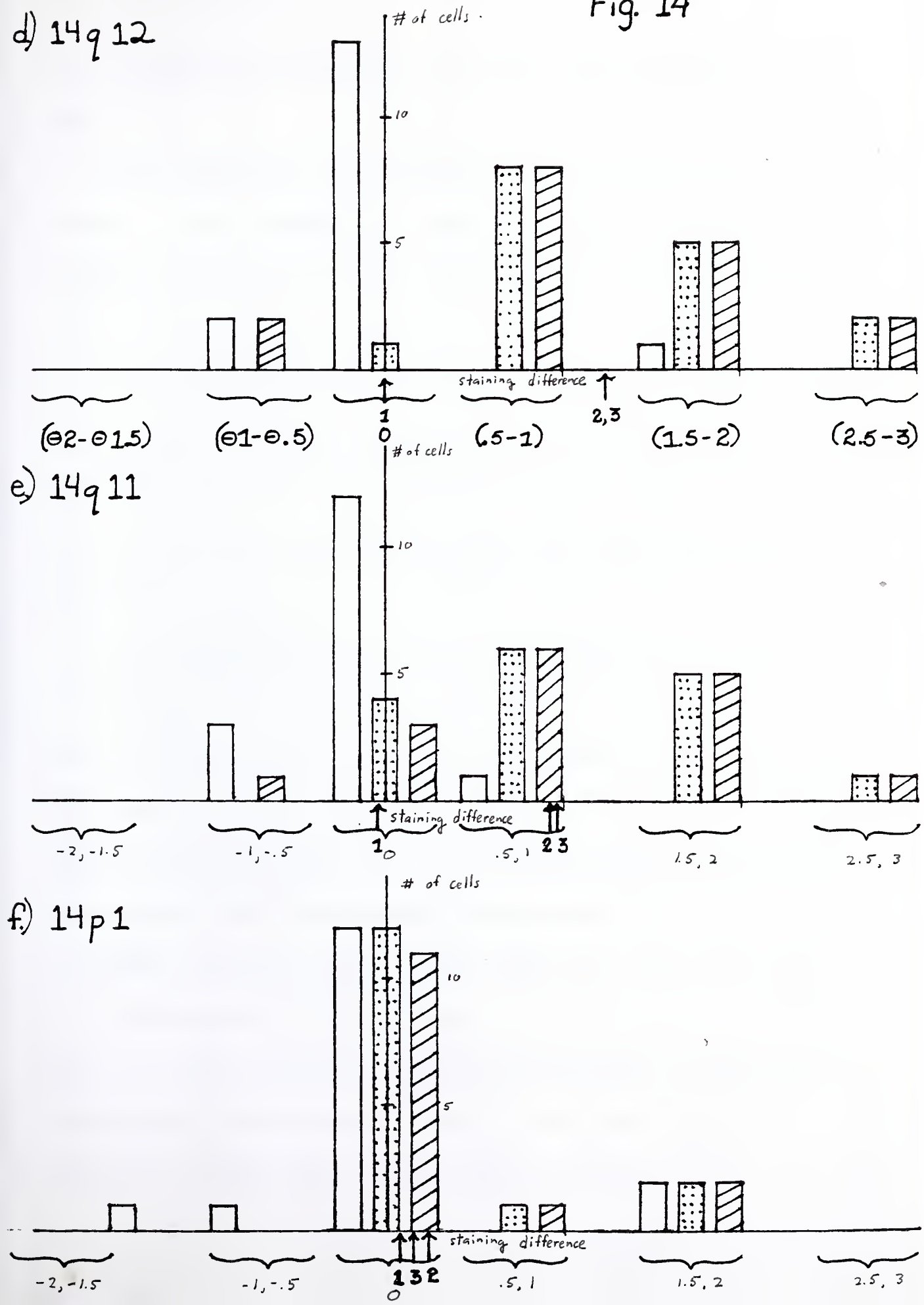

a) 14q31
# of cells
10
5
staining difference
1
0
23
(0-2-0.5)
(0-1-0.5)
(.5-1)
(1.5-2)
(2.5-3)
b) 14q23
# of cells
10
5
staining difference
1
0
32
-2, -1.5
-1, -.5
.5, 1
1.5, 2
2.5, 3
c) 14q21
# of cells
10
5
staining difference
1
0
2,3
1.5, 2
-2, -1.5
-1, -.5
.5, 1
2.5, 3

Fig. 14


d) 14q12
# of cells
10
5
staining difference
1
0
2,3
(0-0.5)
(0.5-1)
(1.5-2)
(2.5-3)
e) 14q11
# of cells
10
5
staining difference
1
0
2
3
-2, -1.5
-1, -.5
.5, 1
1.5, 2
2.5, 3
f) 14p1
# of cells
10
5
staining difference
1
3
2
0
-2, -1.5
-1, -.5
.5, 1
1.5, 2
2.5, 3

band was paler in $14^{X}L$ than in the other two chromosomes in all cells.

Band 14q21 had a mean ($14^{X}E$-$14^{X}L$) of 1.78 and a mean (14-$14^{X}L$) of 1.78. These differences are statistically significant. One of the 16 cells showed 14q21 to be of equal or greater intensity in the $14^{X}L$ than in the $14^{X}E$ or the 14.

Band 14q12 had a mean ($14^{X}E$-$14^{X}L$) of 1.28 and a mean (14-$14^{X}L$) also of 1.28. These differences are statistically significant. In 2/16 cells, the 14q12 band of the $14^{X}L$ had an equal or greater intensity than the 14q12 band of one of the other two analyzed chromosomes.

Band 14q11, the quinacrine-dull band which is the band of 14q closest to the centromere, was more palely stained on $14^{X}L$ than on $14^{X}E$ and on 14 in a lesser proportion of cells than were the quinacrine-bright bands. The mean ($14^{X}E$-$14^{X}L$) was 0.91 and the mean (14-$14^{X}L$) was 0.88. The differences are statistically significant. 5/16 cells showed 14q11 on the $14^{X}L$ to be of equal or greater intensity than 14q11 on either the $14^{X}E$ or the 14.

14p1 had mean ($14^{X}E$-$14^{X}L$) equal to 0.17 and mean (14-$14^{X}L$) equal to 0.26. Neither of these differences are statistically significant. In 14/16 cells, 14p1 was of equal or greater intensity in the $14^{X}L$ than in the $14^{X}E$ or in the 14.

In order to determine the degree of spreading of late replication in each $14^{X}L$ chromosome, the above data were organized in the following way. In an analogous fashion to what was done for the T pulse, a diagram was made of 15 of the 16 cells studied (one metaphase did not contain a suitable control $14^{X}E$); bands exhibiting discrepancies in staining intensity between the $14^{X}L$ and the $14^{X}E$ are indicated (figure 15). A difference of 1.5 scoring units was defined to be the minimum difference indicating a significant disparity between the staining intensities of two bands. This tolerance was chosen instead of the 1.0 used in the T pulse because the mean ($14^{X}E$-$14^{X}L$) over the four bands 14q31, 14q23, 14q21, and 14q12 is 1.62 for the B pulse whereas it is 0.97 for the T pulse, i.e. because a discrepancy of 1.5 in the B pulse is equivalent to a discrepancy of 1.0 in the T pulse.

The extent of spreading for each cell was determined from this diagram according to the same rules used for the T pulse. These rules are applicable because the B pulse was given during approximately the same phases of the cell cycle as was the T pulse. This is known to be so because:

a) an equivalent cell splitting and labeling protocol was followed

b) the B pulse data is consistent with such a scheme for the

timing of labeling: BrdU seems to have been introduced after the completion of the replication of band 14q12 and band 14q23 on the $14^{X}E$ chromosome in all cells (during or after labeling time 2 in figure 11) since in no cell was band 14q12 or band 14q23 pale on $14^{X}E$. It may be inferred that in some cells $14^{X}E$ was exposed to BrdU after the termination of replication of its 14q12 and 14q23, at the time just before the termination of replication of its 14q21 and 14q31 (at time 3 in figure 11). This inference is based on the fact that some cells have all bands of $14^{X}L$ lighter staining than those of $14^{X}E$ (indicating that each region of the $14^{X}E$ was labeled after most or all of its DNA had replicated) while also showing band 14q21 of $14^{X}E$ staining more palely than the other bands of that chromosome (indicating that some portions of 14q21 had replicated in the presence of label). However, no cells had label introduced after the <u>completion</u> of replication of bands 14q21 and 14q31 on $14^{X}E$ (i.e. at time 4 or later of figure 11) since the $14^{X}E$ chromosomes in all cells had relatively pale staining of band 14q21. Thus the label in B pulse, like the label in T pulse was introduced during times near 2 and 3 of figure 11.

Band 14p1 was omitted from the analysis as it was in the T pulse.

TABLE 3

Band	Mean difference	s_p	95% confidence interval	accept H_o?
14q31				
a) $14-14^X_E$	.06	0.74	-.47 to .59	Yes
b) $14-14^X_L$	1.44	0.69	.94 to 1.94	No
c) $14^X_E-14^X_L$	1.50	0.81	.92 to 2.08	No
14q23				
a) $14-14^X_E$	.03	0.20	-.11 to .17	Yes
b) $14-14^X_L$	1.94	0.62	1.49 to 2.39	No
c) $14^X_E-14^X_L$	1.91	0.64	1.45 to 2.37	No
14q21				
a) $14-14^X_E$	0	0.61	-.44 to .44	Yes
b) $14-14^X_L$	1.78	0.52	1.40 to 2.16	No
c) $14^X_E-14^X_L$	1.78	0.66	1.30 to 2.26	No
14q12				
a) $14-14^X_E$	0	0.33	-.24 to .24	Yes
b) $14-14^X_L$	1.28	0.70	.77 to 1.79	No
c) $14^X_E-14^X_L$	1.28	0.53	.90 to 1.66	No
14q11				
a) $14-14^X_E$	.03	0.31	-.19 to .25	Yes
b) $14-14^X_L$	.88	0.54	.49 to .72	No
c) $14^X_E-14^X_L$	.91	0.58	.76 to 1.06	No
14p1				
a) $14-14^X_E$	.09	0.77	-.46 to .64	Yes
b) $14-14^X_L$	.26	0.77	-.31 to .83	Yes
c) $14^X_E-14^X_L$	.17	0.77	-.40 to .74	Yes

The composite diagrams of chromosomes subjected to the B pulse (figure 15) yield the following results (see table 4). Discounting indeterminate cells, spreading extends to band 14q31 in 100% of cells, to band 14q23 in 80%, to band 14q21 in 79%, to band 14q12 in 47%, and to band 14q11 in 43%. 95% confidence intervals for these percentages for n = 15 are: 14q31: 79-100%, 14q23: 53-95%, 14q21: 52-95%, 14q12: 24-75%, and 14q11: 37-70%.

Ag Stain:

Nine cells stained well with the Ag stain method and were analyzed. The cells fell into three classes with respect to Ag-staining of the 14^Xs. There were 3 cells in each class.

The first category consists of cells showing a moderate-heavy amount of stain on both 14^Xs. An example of such a cell is shown in figure 16. The second group of cells showed a moderate-heavy amount of stain on one 14^X but light-absent staining on the other (see figure 17). The last category of cells showed light-absent staining of both of the 14^Xs (see figure 18).

In order to quantitate the above finding, the acrocentric chromosomes of each cell were scored for Ag-stain size. Figure 19 indicates the quantitative scoring of the staining on the 14^Xs for each of the 9 cells. In order to determine whether staining discrepancies from cell to cell were the result of staining artifact,

Figure 15: The extent to which staining difference between 14^{X}_{E} and 14^{X}_{L} has spread based upon the B pulse data. 15 14^{X}_{L} chromosomes are diagrammed. Boxes containing parallel diagonal lines indicate bands which are at least 1.5 staining units lighter on 14^{X}_{L} than on 14^{X}_{E}. Single diagonal slashes indicate bands which were expected to stain 1.5 units lighter on 14^{X}_{L} than on 14^{X}_{E} based upon the staining differences at other bands but which were actually only 1 unit lighter on 14^{X}_{L} than on 14^{X}_{E}. Vertical red lines show the chromosomal segments in which spreading of late replication must have terminated.

Fig. 15

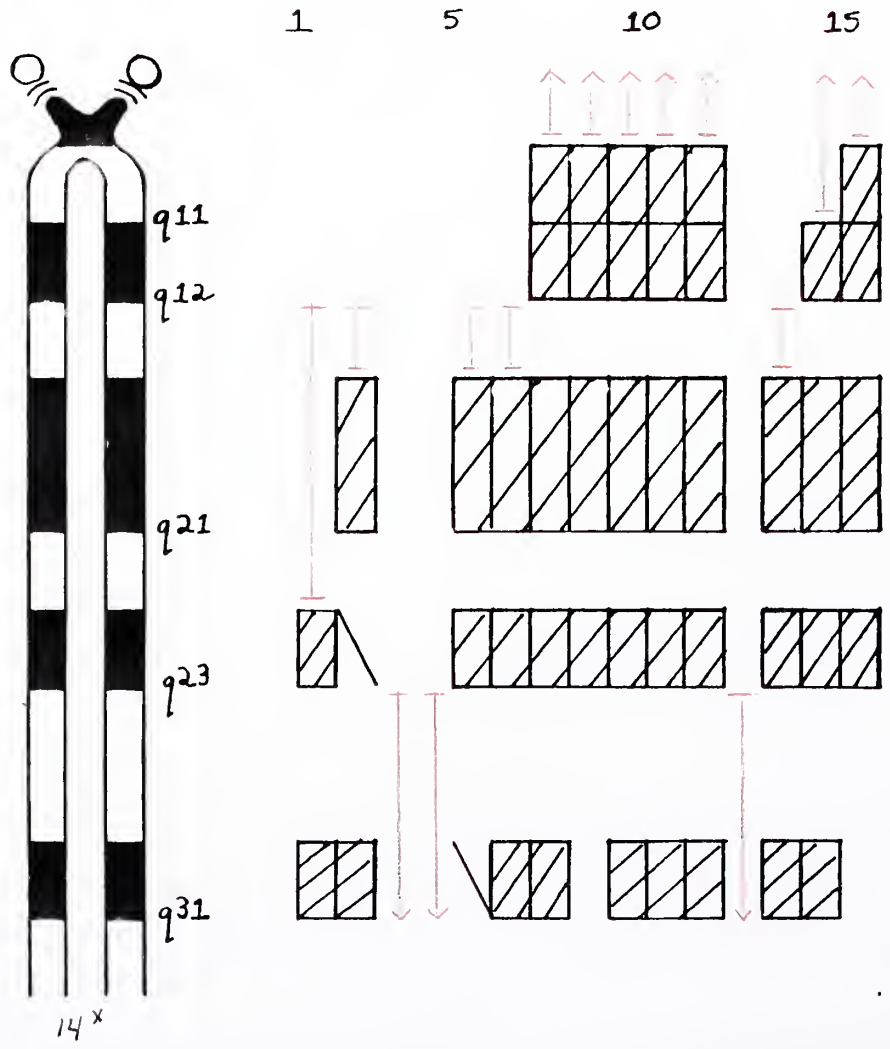


TABLE 4: Spreading of late replication in B Pulse

Band	# of cells with band inactive	#of cells with band active	#of indeterminate cells	% late replicating	% neglecting indeterminate cells
14q31	12	0	3	80-100	100
14q23	12	3	0	80	80
14q21	11	3	1	73-80	79
14q12	7	8	0	47	47
14q11	6	8	1	40-47	43

the Ag stain size of the acrocentrics other than the 14^Xs in cells from each of the three categories cited above were compared. Figure 20 presents this comparison. One of the two 13's, one of the two 21's, and both 22's were moderately to heavily stained (had scores of 2-3) in cells in all of the three categories described above. The 14, one 15, and one of the 21's were lightly stained (score of 0-1) in cells of all categories. The remaining chromosomes showed greater variation. The homologue pair that showed the clearest difference in staining based upon cell category was the 15's which had an average size of 2 when both 14^Xs were moderately-heavily stained, of 1.75 when one 14^Xstained "moderate-heavy" and the other stained "light-absent," and of 0.67 when both of the 14^Xs stained "light-absent".

Mean Ag stain sizes for each of the acrocentrics were calculated and the result is presented in figure 21 a).

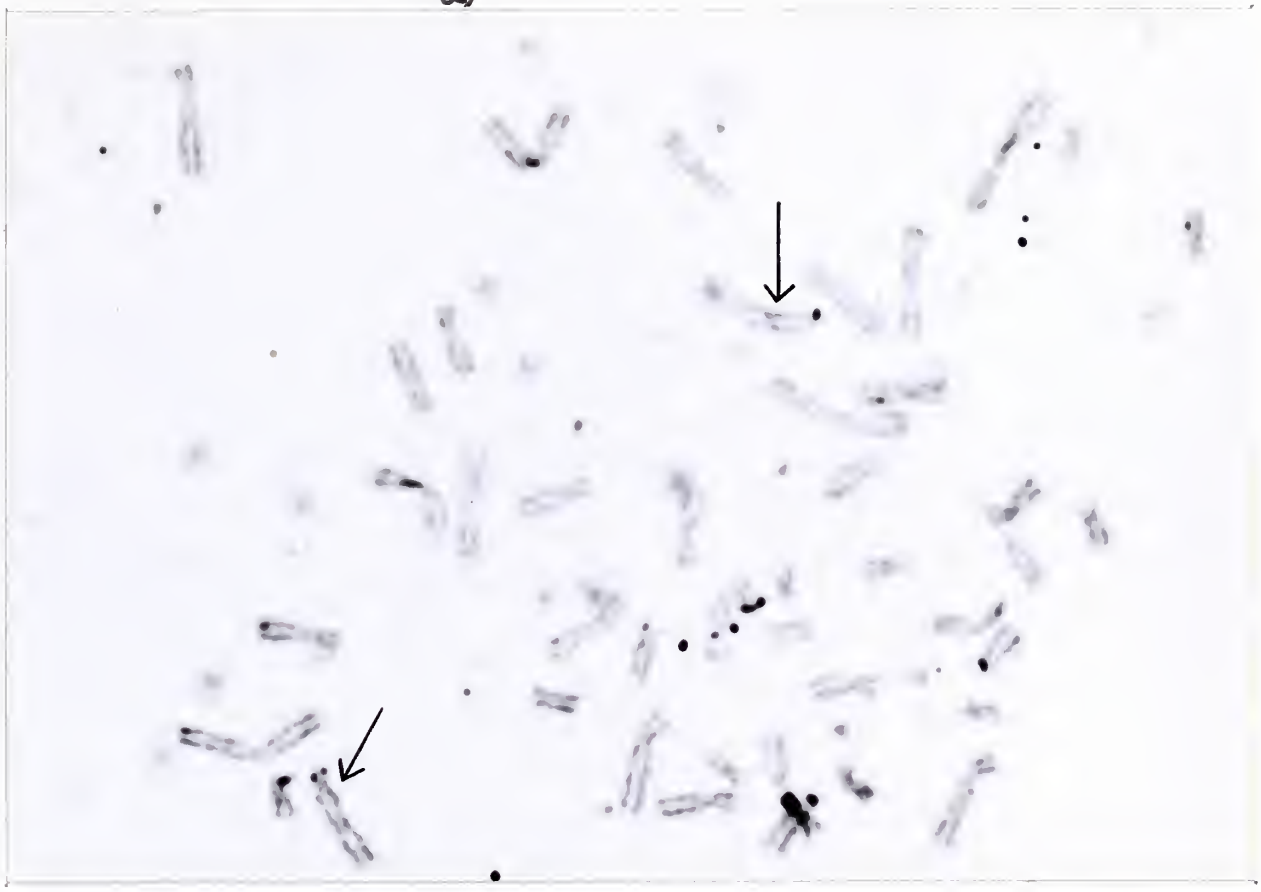
Satellite Associations:

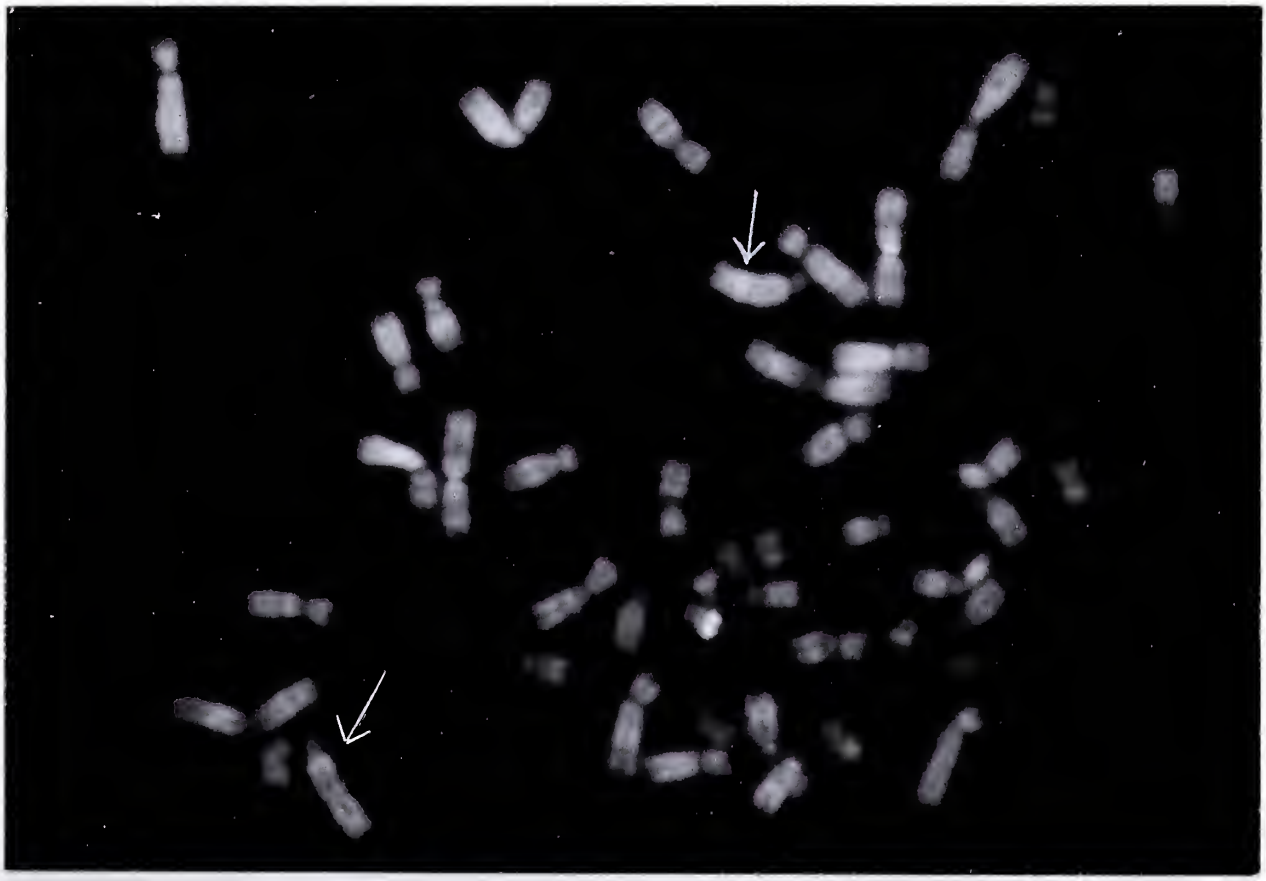
The mean number of satellite associations per cell made by each of the acrocentrics is shown in figure 21 b) and c). The mean number of associations/cell was 2.25 for the T pulse, 0.63 for the B pulse, and 2.67 for the Quinacrine/Silver technique. In the B pulse, most associations involved chromosomes which could not be identified. The B pulse associations were therefore omitted

Figures 16-18: Three patterns of silver staining. Part a) of each figure demonstrates Ag-staining and part b) quinacrine staining of the cell photographed in a). Arrows indicate the 14^X chromosomes. In figure 16, both 14^Xs have "moderate-heavy" Ag-staining. In figure 17, one 14^X shows "moderate-heavy" Ag-staining and the other shows "light-absent" staining. In figure 18, both 14^Xs have "light-absent" staining.

Fig. 16

a)

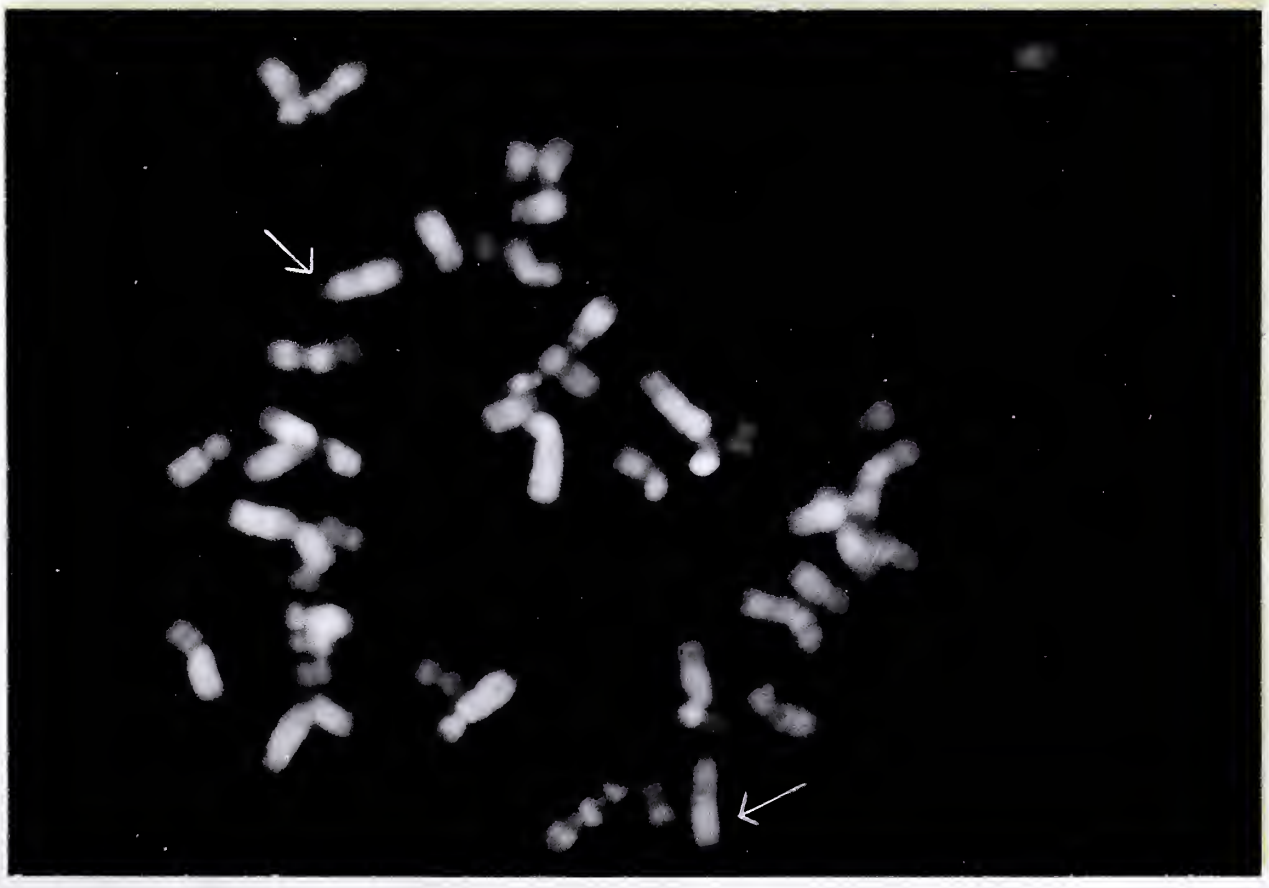




b)

Fig. 17

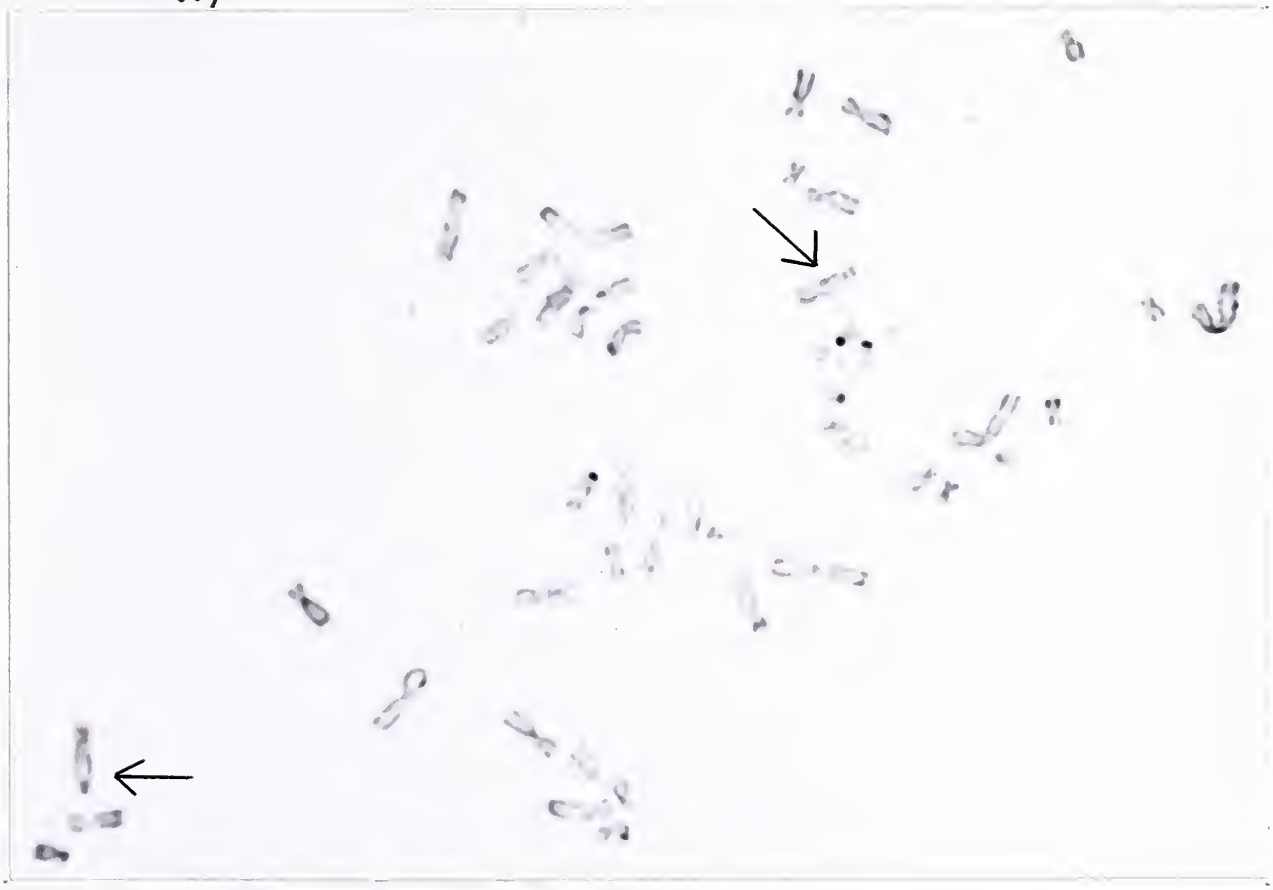
a)

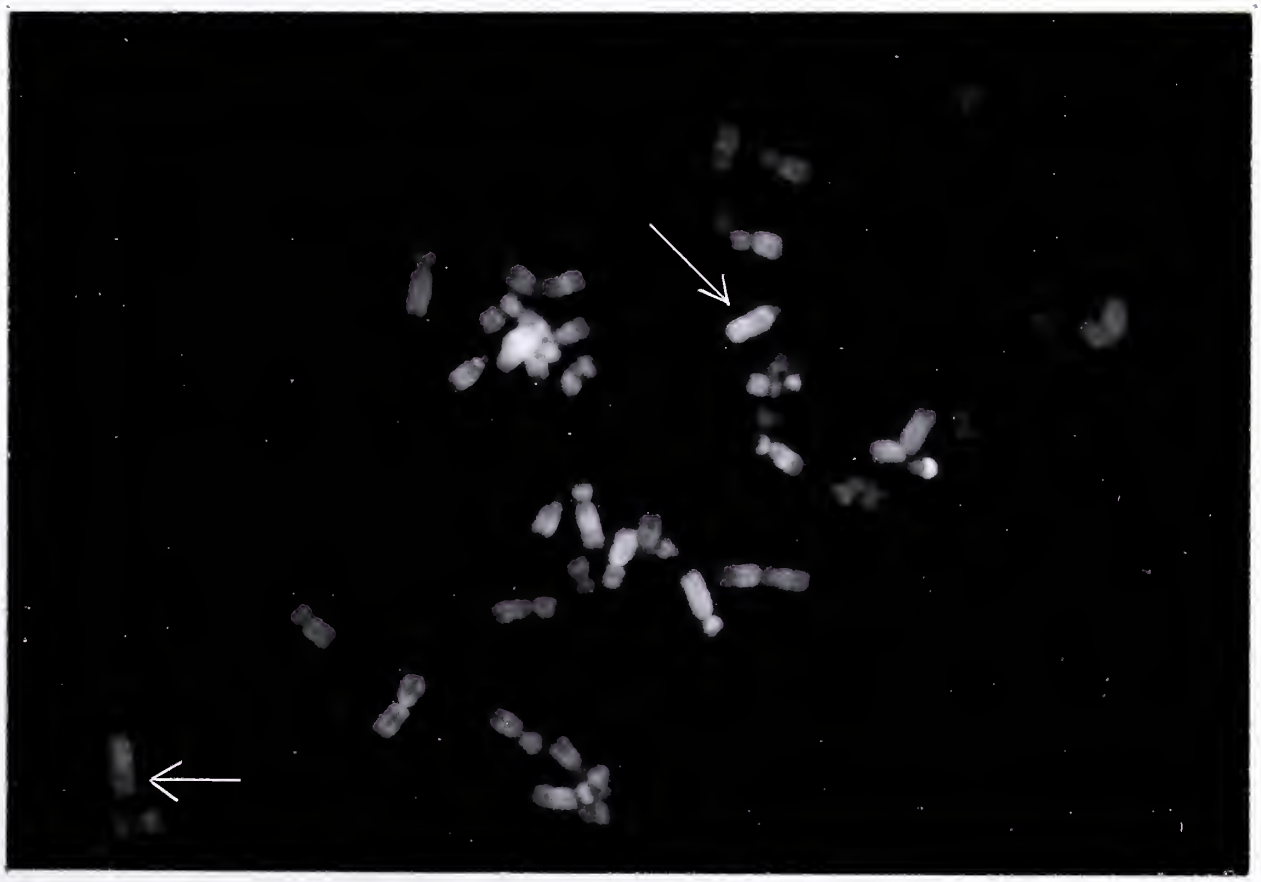


b)

Fig 18

a)





b)

from the analysis so as not to skew the calculated frequencies towards the chromosomes which are morphologically identifiable in the B pulse.

Figure 2 (arrow) shows an association involving the $14^{X}E$ and figure 22 (arrowhead) shows one involving the $14^{X}L$. In the metaphase plates in which $14^{X}E$ could be distinguished from $14^{X}L$, the $14^{X}E$ was in association 10 times and the $14^{X}L$ was in association 7 times. This difference is not statistically significant at $p < .05$ by chi-square. Thus, the $14^{X}L$ associated 30% less than did the $14^{X}E$ but our small sample size precluded an assessment of whether this difference was real or merely reflected chance variation. Of the metaphases in which the early and late 14^{X}s could not be distinguished, 4/36 had both 14^{X}s in association.

It can be seen from figure 21 that there was a good correlation between the frequency of satellite association of each pair of homologous chromosomes and the mean Ag-stain size of the pair. With regard, specifically, to this correlation in the 14^{X}s, it was seen that the 14^{X}s with moderate-heavy silver stain were in association twice whereas the 14^{X}s with light-absent stain were involved in no associations.

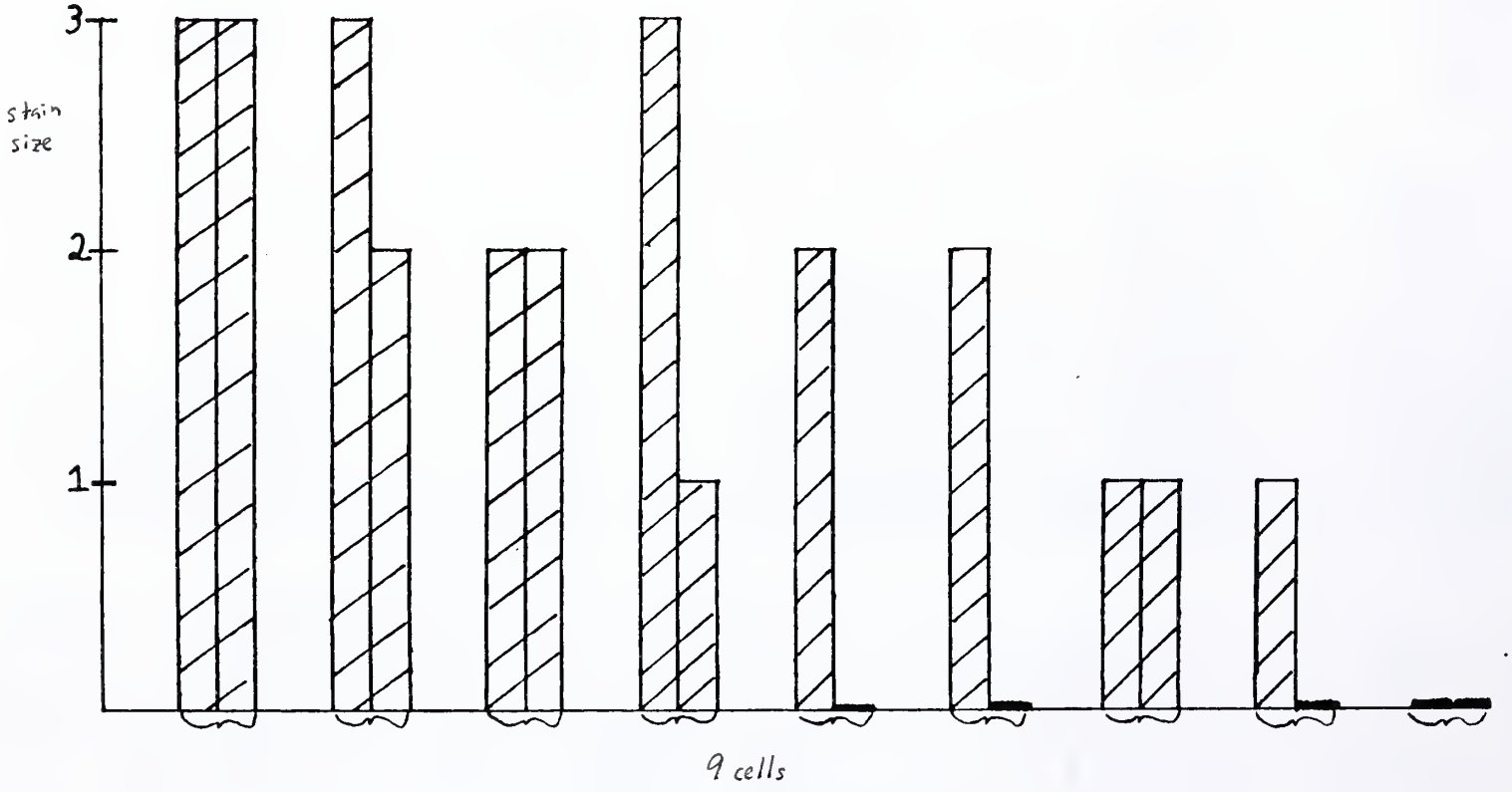
Figure 19: Ag stain size of the 14^Xs in 9 cells. The crosshatched bars represent the 14^Xs in each cell. Each cell is set apart from the others by brackets. The ordinate indicates Ag-stain size on the 0-3 scale described in the text. Cells are arranged in order of decreasing total Ag-stain on the 14^Xs.

Figure 20: Ag-stain size of the acrocentrics other than the 14^Xs in cells of categories 1,2, and 3 (see text). The ordinate indicates Ag-stain size on the 0-3 scale. For each graph, bars filled-in in the same way indicate chromosomes from the same cell.

Figure 21: A comparison of the mean Ag-stain size of each acrocentric with the number of satellite associations made by that acrocentric. Part a) shows mean Ag-stain size using the 0-3 scale. Part b) shows mean number of satellite associations per cell for the T pulse. Part c) shows the mean number of satellite associations for the Ag/quinacrine techniques.

Fig. 19

Ag-Stain Size of the 14^{x}s in 9 Cells



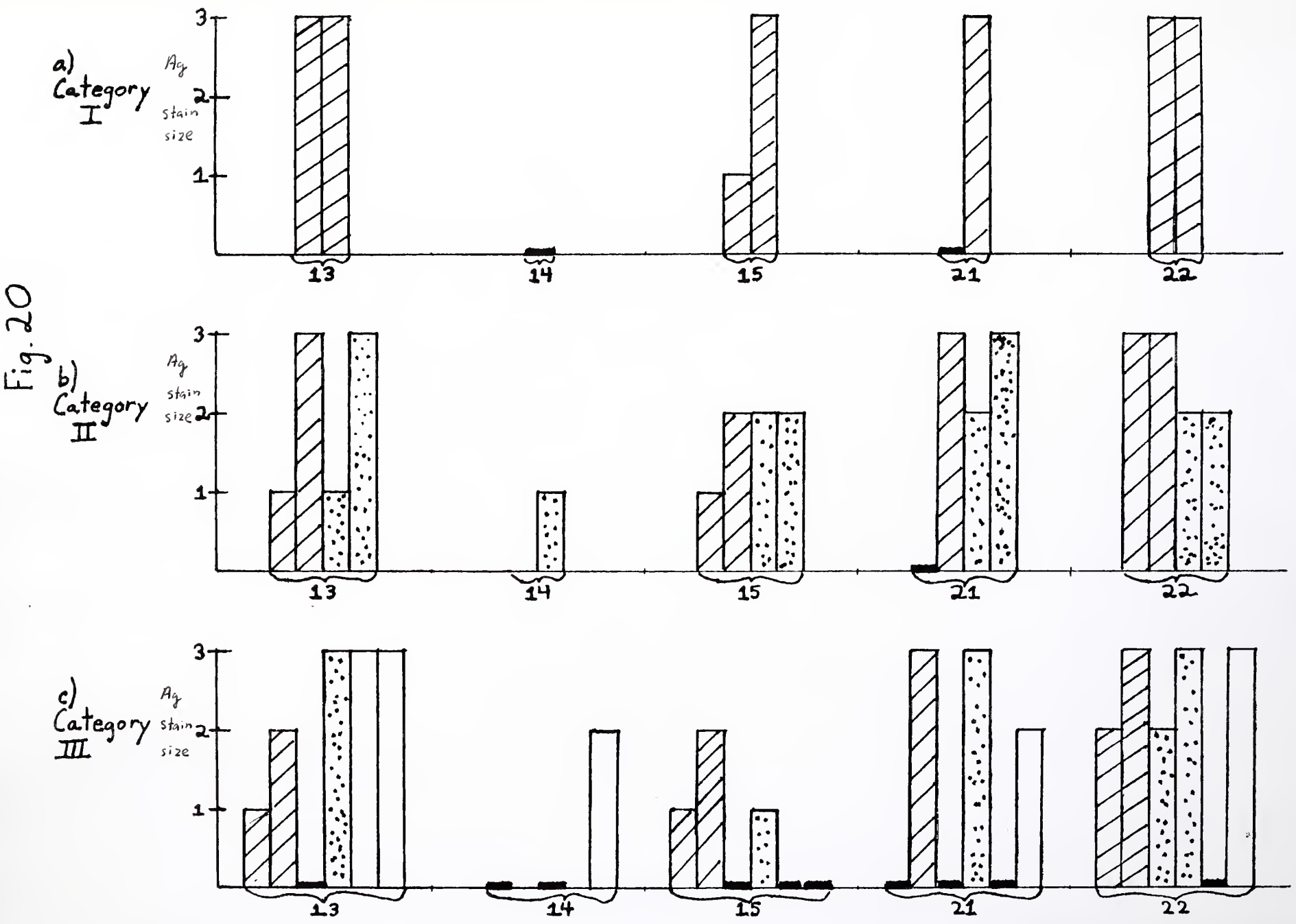
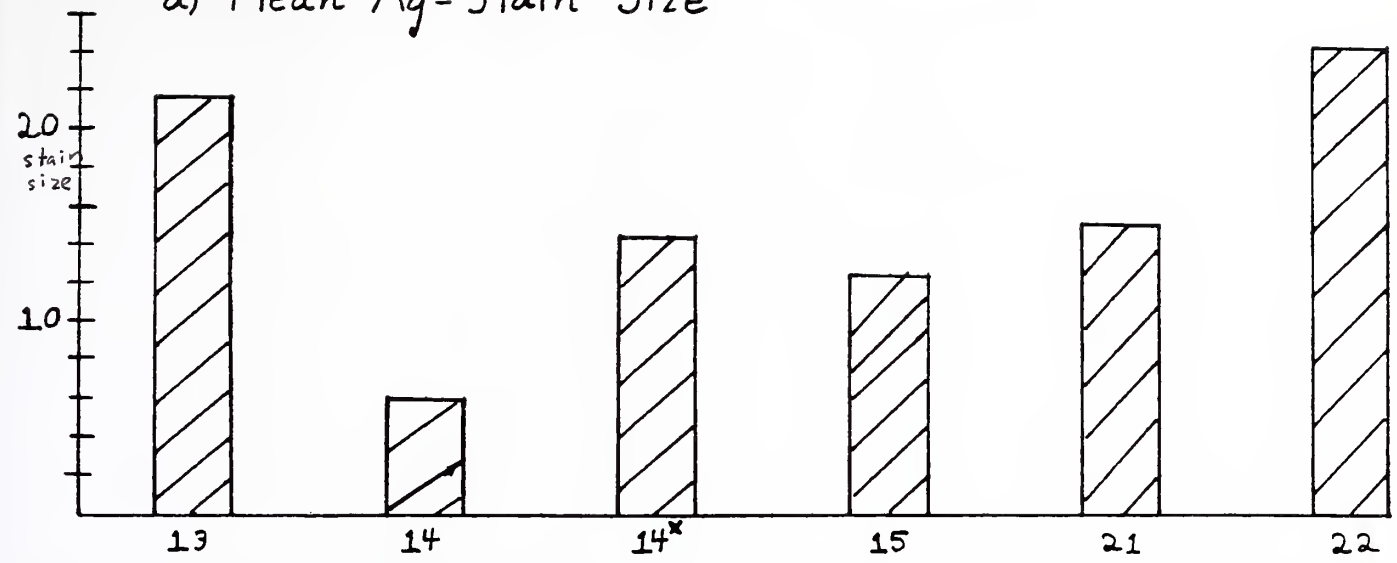

a)
Category
I
Ag
stain
size
3
2
1
13
14
15
21
22
b)
Category
II
Ag
stain
size
3
2
1
13
14
15
21
22
c)
Category
III
Ag
stain
size
3
2
1
13
14
15
21
22

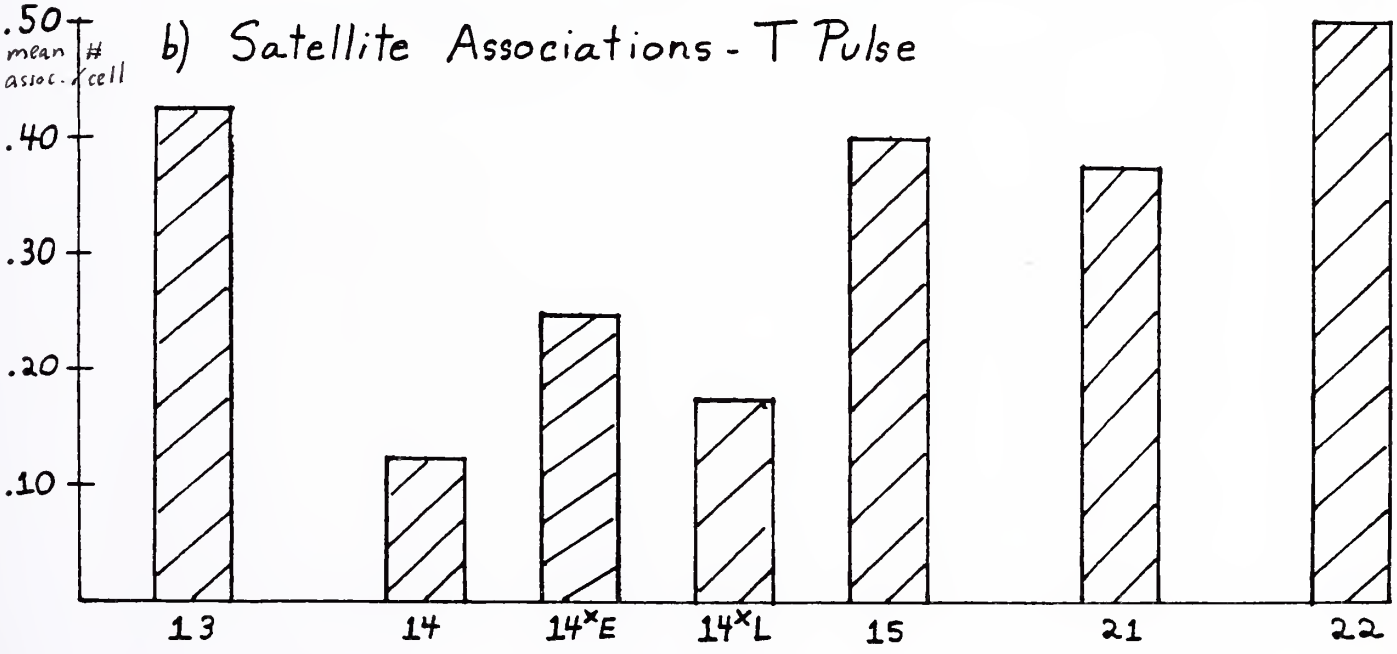
Fig. 20

Fig. 21

a) Mean Ag-Stain Size



b) Satellite Associations - T Pulse



c) Satellite Associations - Ag/Quinacrine

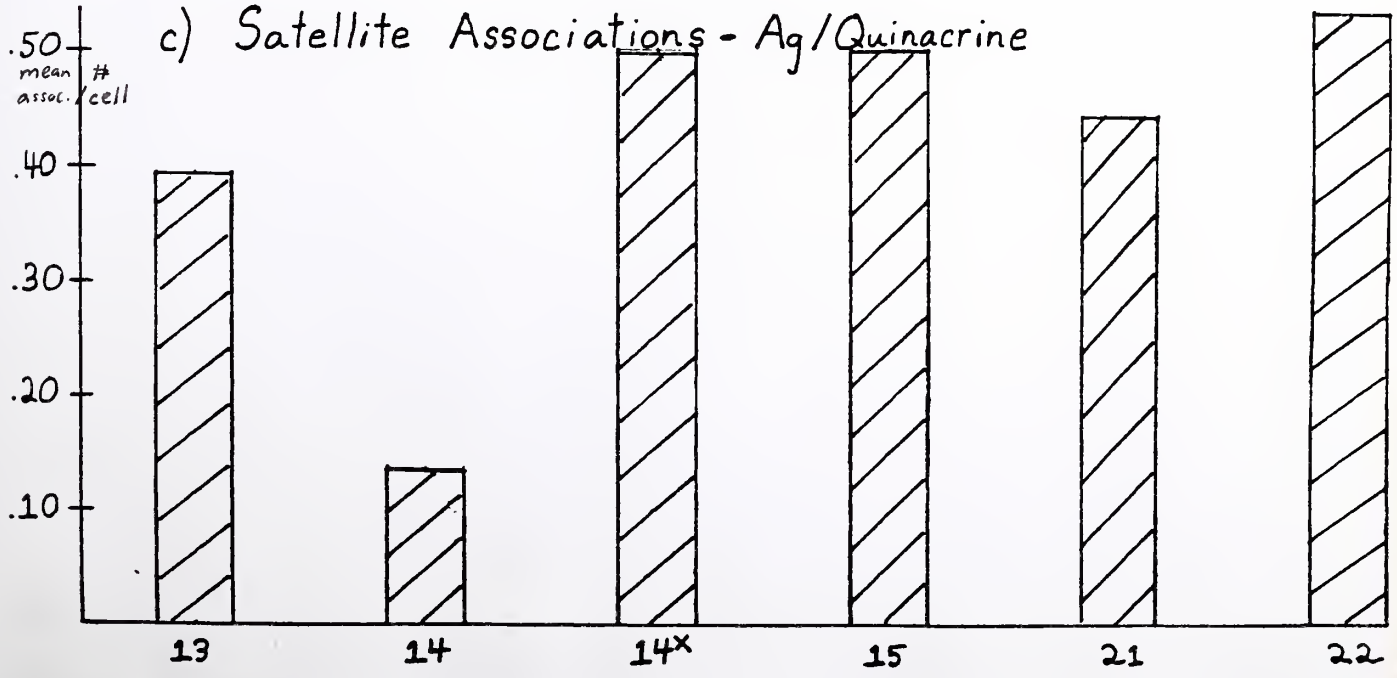


Figure 22: A T pulse metaphase showing a satellite association between $14^{X}L$ and chromosome 15 (arrowhead).

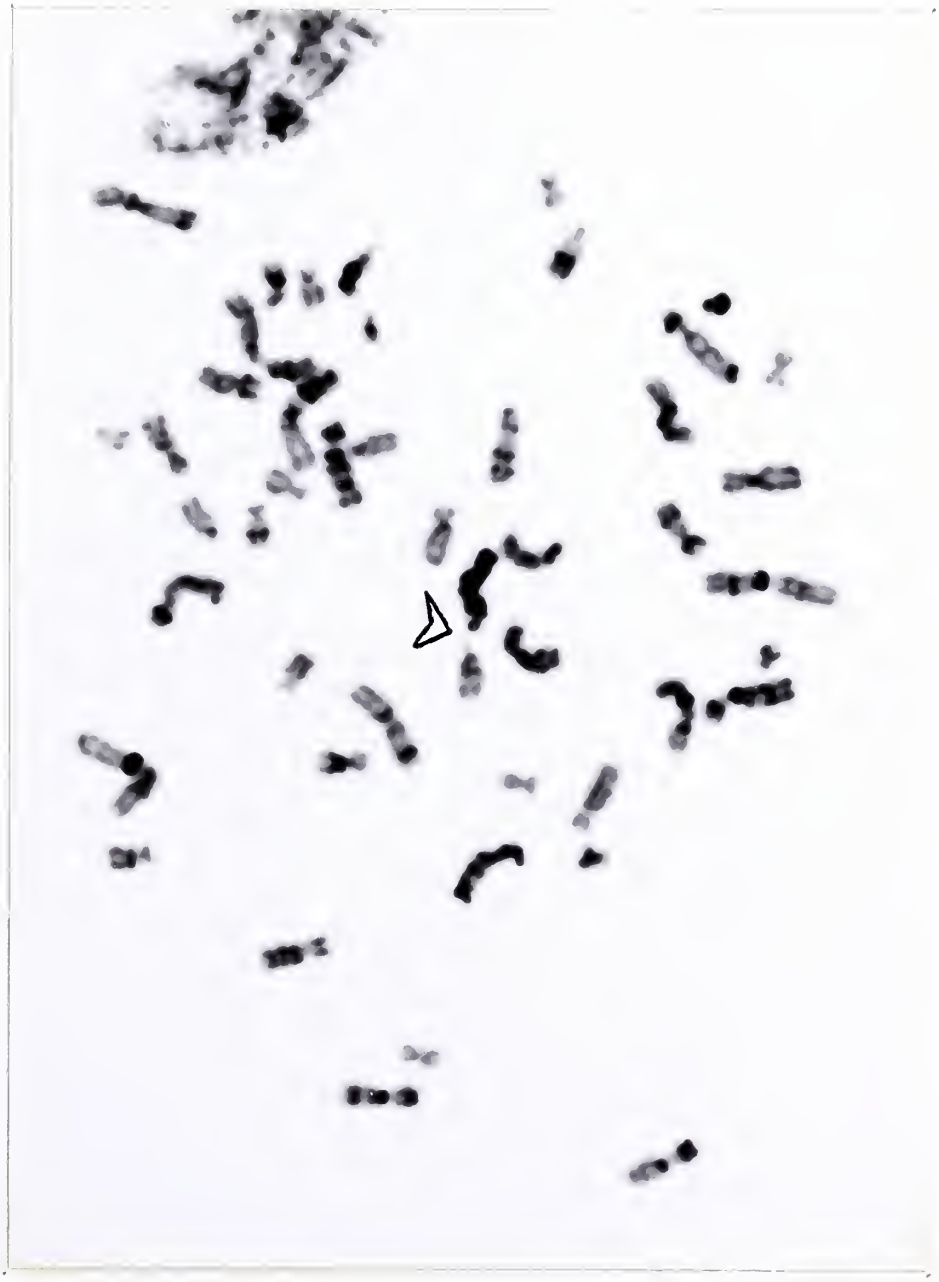


Fig. 22

DISCUSSION

Taken as a whole, the data of this experiment indicate that inactivation on the $14^{X}L$ chromosome of the KOP-2 translocation extends beyond the X portion of the chromosome to a variety of levels on the 14 portion. Each method used to examine the degree of spreading yielded information about different aspects of the phenomenon of termination.

The T pulse technique yielded metaphases with good quality banding which demonstrated termination of replication asynchrony between the two 14^{X} chromosomes at several levels. A quantitative assessment of the staining intensity of four chromosomal bands made possible a tabulation of the number of cells exhibiting each spreading pattern. This tabulation indicated that the number of cells in which an autosomal region proximal to the centromere of $14^{X}L$ was inactivated decreased with the distance of that region from the X portion of the chromosome. Evaluation of spreading beyond the centromere was technically unsatisfactory in the T pulse.

A few points of special interest concerning the T pulse deserve comment. First, it should be recalled that in the analysis of the T pulse data, autosomal bands on certain $14^{X}L$ chromosomes were said to be later replicating than the corresponding bands

on the 14^{X}_{E} chromosomes in circumstances in which the bands on both chromosomes were positively stained but the bands were more intensely stained on the 14^{X}_{L} than on the 14^{X}_{E}. This is a somewhat unusual way of documenting replication asynchrony in studies of this kind and should be identified as such. Usually, only the presence of a band in the late replicating chromosome and its absence in the early replicating chromosome is judged to be sufficient evidence for replication asynchrony. It must be recognized, however, that the KOP-2 translocation is a special case since the two chromosomes being compared are identical, not just homologous, and the expectation is that staining intensities be identical. Thus, a difference in band intensity (reflecting a greater proportion of the darker band replicating in the presence of thymidine than of the paler band) sufficed to show that there was a significant difference in the time of replication between the two involved bands.

Secondly, it is interesting that the T pulse showed differential labeling for bands which stain quinacrine-dull as well as bands which stain quinacrine-bright. In the T pulse a Q-dull autosomal band on the 14^{X}_{L} was said to be late replicating when it was both a) as dark as adjacent late replicating Q-bright bands and b) significantly darker than the corresponding Q-dull band on 14^{X}_{E}.

Using these criterea, the Q-dull band 14q22 was late replicating in 16 T pulse cells, 14q13 in 13 cells and 14q11 in 6 cells. Late replication in these bands was always consistent with the spreading pattern deduced from the staining of the Q-bright bands. It has been previously shown that, as a rule, quinacrine-dull bands start replicating before quinacrine-bright bands do and finish replicating before quinacrine-bright bands do (66). The discussion about thymidine incorporation times for the 36 T pulse cells which appeared in "Results" indicated that thymidine label was added in all cells after the termination of replication of at least two Q-bright bands (14q12, 14q23) on the early replicating chromosomes; this implies, according to the above statement, that thymidine was introduced long after the termination of replication of the Q-dull bands on these early replicating chromosomes. This being so, the heavily thymidine labeled Q-dull bands which appeared on late replicating chromosomes in our study must have finished replication long after the corresponding Q-dull bands on early replicating chromosomes. The significance of this conclusion is that a) it is the most definitive proof that replication asynchrony exists between bands on early and late replicating chromosomes and b) it indicates the magnitude of the delay in replication timing which takes place in "late" replicating regions of $14^{X}L$.

The T pulse results have enabled us to consider several issues beyond simply a gross estimation of various levels at which spreading may terminate. One important question to be answered is whether late replicating autosomal regions have abrupt borders or do not -- i.e. whether the most distal DNA affected by spreading of late replication is somehow only "partially" affected. In more specific terms, the question may be rephrased: do regions of DNA situated where spreading terminates have a replication time intermediate between "late" and "early" or, alternatively, do these regions contain an interspersal of small "early" and "late" replicating regions? In our study, the way this problem could best be approached was to consider metaphases that had the autosomes and the Y showing distinct banding and to look, in these metaphases, at the distal border of the dark staining area on the $14^{X}L$. If this border had tended to be less distinct (more "fuzzy") than the borders of other dark staining regions in the metaphase, this would have been consistent with the existence of a "partially late replicating" region. No cell in our study demonstrated this phenomenon. Thus our data are supportive of an abrupt termination of late replication.

If spreading does end abruptly, there must be something special about the loci at which termination may occur. Let us

call a locus of this type a "terminator". Our data indicate the minimum number of terminators which must exist on the autosomal segment of $14^{X}L$. Based upon the spreading patterns described in the "Results" section, the T pulse (and B pulse) data show that there must be at least one terminator proximal to band 14q23 and one proximal to band 14q12. There probably is a terminator distal to band 14q12, as well, since cells labeled at equivalent portions of the cell cycle which have spreading to the level of 14q12 show differential staining between the 14^{X}s according to <u>two</u> major patterns. The first has extension of dark stain just to the centromeric border of 14q12. The second shows extension of dark stain to the tip of 14p. Thus, there are most likely at least three terminators on the autosomal segment of $14^{X}L$.

The question next suggested is whether an upper limit for the number of terminators can be arrived upon from our data.

Essentially this problem boils down to the question of whether terminators may occur "anywhere" or whether there is some restriction on their position on $14^{X}L$. The hypothesis best suited to testing using our own results is that terminators may occur only at band borders, never in the middle of bands. Stated another way, this hypothesis says that Paris Conference designated chromosomal bands are late replicating in an all or none fashion.

The staining patterns of the $14^{X}L$ chromosomes in many metaphases seem to be consistent with this hypothesis. For example, in cell b) of figure 5, the material on $14^{X}L$ which is darker than that on $14^{X}E$ terminates at the distal border of 14q31. Similarly for e) and f) and 14q21, g) and h) for 14q12, and probably for c) and 14q23. However, despite these suggestive examples, the number of variables affecting staining in the T pulse and the limitations inherent in the technique make any conclusions extremely tentative. Such variables and limitations include variable degrees of chromosome contraction, variability in the phase of the cell cycle at which thymidine was added, and the limit of resolution of BrdU labeling (resolution is approximately .3u., the resolution of light microscopy). Thus, the T pulse data do not give an upper limit to the number of terminators on the autosomal portion of $14^{X}L$.

The B pulse data were, on the whole, less informative than the T pulse data. Chromosomes stained in this protocol were more condensed and analysis of banding intensity was hampered. Moreover, it was inherently more difficult to define the limits and the intensity of a small pale band surrounded by two dark bands (many of the late replicating bands in B pulse) than it was to do so for a small dark band surrounded by two pale bands (the corresponding

late replicating bands in T pulse). Lastly, the parameter of labeling time could not be assessed for the B pulse metaphases.

The B pulse confirmed the general scheme of spreading patterns suggested by the T pulse. While the percentage of cells displaying inactivation of each band was not exactly the same in the B pulse as in the T pulse, the relative magnitudes of the decreases in going from a specific band to the band next most distal were the same. The B pulse, like the T pulse showed quinacrine-dull bands with extensive staining differences between tne $14^{X}L$ and the $14^{X}E$. As suggested above, the B pulse data were not suitable for addressing questions about the possible coincidence of late replicating regions and chromosomal bands and about the presence of an abrupt termination to spreading of late replication.

Silver staining was useful in providing information about spreading beyond the centromere of the $14^{X}L$. The most definitive result was shown by the existence of the three cells which showed moderate-heavy staining on both 14^{X}s (the cells of category 1). This proved that there is a population of cells without functionally significant spreading of inactivation to the rDNA of the $14^{X}L$. The question naturally suggested by this is whether spreading of inactivation to the rDNA occurs in <u>any</u> cells of the karyotype. In order to answer this question, it must be decided whether the

light-absent staining on one or both of the 14^Xs in the cells of the other two categories reflects a biological or an artifactual difference between these cells and the three cells cited above.

In the cells of category 2 (cells with one moderate-heavily stained 14^X and one light-unstained 14^X) the best evidence points toward a true biological difference between the differentially stained 14^Xs. Firstly, the 14^Xs in these cells have an identical amount of material which is potentially Ag-stainable (one 14^X is derived from the other). The relatively heavy staining of one of the 14^Xs implies that the metaphase as a whole was stained sufficiently heavily to stain the other 14^X to the same degree. Since it has been shown by D.A. Miller et. al. (65) that there is not much variation in the Ag stain size displayed by a given acrocentric chromosome, the failure of the second 14^X to stain as heavily as the first implies that there is some degree of biological inactivation of the lesser staining 14^X. Secondly, the homologous pairs of acrocentrics which have roughly the same mean amount of Ag stainable material as do the two 14^Xs -- the 15's and the 21's -- do not show a lesser degree of staining in this category of cells than in cells of category 1. This also suggests that the staining difference between the two 14^Xs is not a result of poor staining. Thus, the three cells of category 2 seem to show spreading of

inactivation to the rDNA.

Cells in category 3 (both 14^Xs have light-absent Ag-stain), however, probably have a decreased overall Ag staining and for this reason, inferences about the presence or absence of spreading beyond the centromere in the 14^Xs' of these cells are difficult to make. The reasons for thinking that the degree of staining of these metaphases is less than for the metaphases of categories 1 and 2 are these. First, as mentioned above, the variance about the mean for Ag-staining size of a given chromosome is small given that the chromosome is repeatedly stained under equivalent conditions. Therefore, since the 14^X with the largest Ag stain in these cells has a much smaller stain size (size = 0 or 1) than does the 14^X with the largest Ag-stain in cells of the other categories (size = 2 or 3) a decreased overall staining of the former cells is likely. Second, the mean Ag-stain size for the 15's in cells of category 3 is markedly lower than in cells of categories 1 and 2. (The mean of the 21's is slightly lower). This fact also implies that there is a decreased overall staining. It is likely that the cell in which both 14^Xs have been assigned a stain size of "1" does not have a significant amount of spreading of inactivation to the rDNA. This is so because the cells of category 2 have demonstrated that the size of the Ag-stain is considerably less

on a 14^X with "inactivated" rDNA than on one with "active" rDNA. On the other hand, the presence or absence of inactivated rDNA cannot be known for the cells in which the 14^Xs have stain sizes of 1 and 0 and of 0 and 0. It is concluded from the Ag stain data, therefore, that the rDNA is inactivated in 33-55% of the studied cells. The 95% confidence interval for this range of percentages for n = 9 is 5-85%.

One last important issue is raised by the one Ag stained cell in category 2 which had silver stain sizes of 3 and 1 on the 14^Xs. The fact that one of these 14^X chromosomes shows significantly less staining than the other but does not have an absence of Ag-stain makes one consider the possibility that inactivation can affect some of the rDNA genes without affecting all.

The satellite association data were quite consistent with the Ag stain data. It has already been noted that mean Ag size for each pair of acrocentrics correlates well with its mean number of associations. The 14^XL participated 30% less actively in satellite association than did the 14^XE but it is not clear whether this reduction was due to chance.

It is apparent that data relevant to the activity of the rDNA on the 14^Xs fit reasonably well with the data on replication kinetics. Taken together, the Ag stain and the satellite associa-

tion data suggest that the percentage of cells in which gene inactivation on a 14^X has spread to 14p is roughly equivalent to the number of cells in which late replication has spread to 14q1. This means that our data support the notion that late replication is equivalent to functional inactivation. Further, they support the idea that spreading of inactivation follows a "gradient" principle -- i.e. that inactivation can affect a distal locus only if it affects more proximal loci (see Introduction).

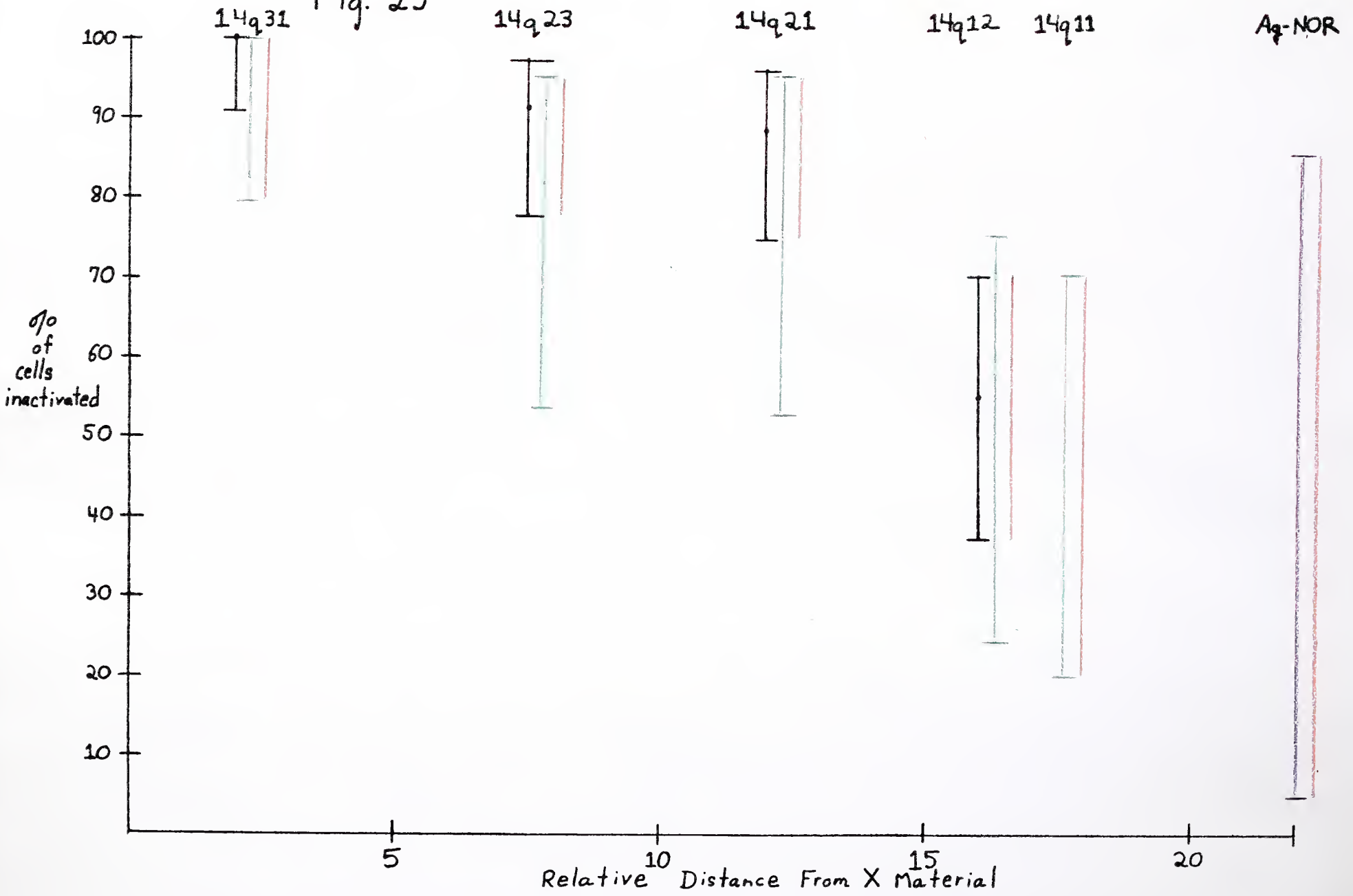
What can be said about the mechanisms that have provided for the spreading patterns described in the above sections? It has been suggested that there are at least 3 terminators in the autosomal segment of the 14^XL. At this point, two parameters in addition to the number and location of "terminators" are considered. The first is the "efficiency" of each terminator. The "efficiency" of a terminator may be defined as the proportion of times that the terminator actually stops the spreading of inactivation when spreading reaches it. Clearly, the greater the efficiency of a terminator, the greater the proportion of cells in which spreading will stop at the level of the terminator rather than go beyond it. The second parameter that must be considered is the degree to which a given spreading pattern is selected against once it has been established in a dividing fetal cell line. The greater the

fitness of a certain spreading pattern, the greater the number of cells that will show this pattern in the adult organism. In general, it is quite difficult to satisfactorily separate out the relative contributions of selection, terminator efficiency, and terminator number. Nonetheless, our data permit some conclusions about the role of these factors in KOP-2.

In the $14^{X}L$ of KOP-2, the drop off in percentage of cells inactivated at a given region with the distance of the region from the X derived material is not linear. This is shown in figure 23 using data from B pulse, T pulse and Ag-staining. The red lines in this figure indicate 90% confidence intervals for the percentage of cells inactivated at each autosomal region. Figure 23 demonstrates that inactivation spreads less easily from band 14q21 to band 14q12 than it does from 14q31 to 14q23 or from 14q23 to 14q21. Given the fact that the most balanced gene expression for this karyotype is achieved when the <u>entire</u> 14 segment is inactivated on $14^{X}L$, it is probable that inactivation distal to band 14q21 actually gives a selective <u>advantage</u>. Thus, the barrier to spread between 14q21 and 14q12 is probably even greater than it appears to be. Therefore, it seems that either the number or the efficiency of terminators is significantly greater between bands 14q21 and 14q12 than more proximally.

Figure 23: The relation of a locus's probability of inactivation to its location on $14^{X}L$. The percent of cells in which a given locus of the $14^{X}L$ is inactivated is plotted against the relative distance of that region from the X portion of the chromosome. The distances are estimated from Paris Conference diagrams of chromosome 14. Black vertical lines indicate 95% confidence intervals from the T pulse data. Green vertical lines indicate 95% confidence intervals from the B pulse data. The purple vertical line indicates the 95% confidence interval from the Ag-stain data. Red vertical lines indicate a combined confidence interval of $\geq$ 90%.

Fig. 23


14q31
14q23
14q21
14q12
14q11
Ag-NOR
100
90
80
70
60
50
40
30
20
10
% of cells inactivated
5
10
15
20
Relative Distance From X Material

It should be noted that our sample size was too small (the 95% confidence interval was too large) for determining whether the secondary constriction (or rDNA per se) had any "blocking effect" above and beyond what occurred at other levels of the chromosome.

All of this discussion relates to the fundamental question about why spreading of inactivation may terminate within autosomal regions but does not seem to terminate within the DNA of the "normal" inactive X. If terminators are as ubiquitous in autosomal material as our study suggests, why are they not present (or functional) on the X? One way to answer this is to conceive of the X chromosome as a structure specifically set up to permit spreading. This would go along with Ohno's observation of the conservation of the intact X chromosome throughout millions of years of speciation (4). In this vein, Eicher's hypothesis about inactivation units spread along the X (see Introduction) is appealing. It not only explains why spreading sometimes extends to the autosomal portion of an X-autosome translocation chromosome and sometimes does not, but also why inactivation so uniformly extends from one end of the inactive X to the other.

In conclusion, this study has made several contributions to the understanding of X-autosome translocations. It has shown that there is a functional measure of gene inactivation -- the Ag-stain --

which correlates reasonably well with the measurement of inactivation by replication kinetics. It has shown that there are at least two and probably three autosomal terminators on the $14^{X}L$ chromosome of KOP-2 and that the degree of inactivation of an autosomal locus on this chromosome is not directly proportional to the distance of the locus from the X segment of the chromosome. Lastly it has suggested that termination of inactivation is abrupt and that, therefore, there exist specialized "terminator" loci on the autosomal segment of $14^{X}L$.

Further work is suggested by this study. In order to determine the incidence of multiple autosomal terminators, karyotypes in which a "partial" degree of spreading has been suggested could be carefully studied with BrdU. In order to see if there is a consistent relationship between "terminators" and the borders of chromosomal bands, suitable karyotypes could be studied with prometaphase spreads and with initial as well as terminal labeling. In order to clarify the role of selection, a chromosome exhibiting "partial" spreading in the mouse could be placed, by various matings, into different known genetic environments. Lastly, in order to test various hypotheses about the molecular mechanism of X inactivation, rDNA could be isolated from karyotypes which exhibit inactivated rDNA and various assays (e.g. for methylation, for associated

proteins, and for sequence) could be made of this very specialized isolate.

REFERENCES

1. Bridges,C.B., "The origin of variations in sexual and sex-limited characters," Am. Natur. 56: 51-63, 1972.
2. Muller, H.J., B.B. League, C.A. Offermann, "Effects of dosage changes of sex-linked genes and the compensatory effect of other gene differences between male and female," Anat. Rec. 51(suppl.): 110, 1931.
3. Gartler, S.M., R.J. Andina,"Mammalian X-Chromosome Inactivation," Adv. Human Genetics 7: 99, 1976.
4. Ohno, S., Sex Chromosomes and Sex Linked Genes, Springer-Verlag, Berlin, 1967.
5. Luchesi, J.C.,"Dosage Compensation in Drosophila," Annu. Rev. Genet., 7: 225, 1973.
6. Ohno,S., C. Stenius, L. Christian,"The XO as the normal female of the creeping vole (Microtus Oregoni)," Chromosomes Today, C.D. Darlington and K.R. Lewis, eds., Oliver and Boyd, Edinburgh, 1966.
7. Lyon, M.F., "Gene Action in the X Chromosome of the Mouse," Nature 190: 372, April 22, 1961.
8. Davidson, R.G., H. Nitowsky, B. Childs,"Demonstration of two populations of cells in the human female heterozygous for glucose-6-phosphate dehydrogenase variants," Proc. Natl. Acad. Sci., USA 50: 481, 1963.
9. Barr, R.L., E.G. Bertram,"A morphological distinction between neurones of the male and female and the behavior of the nucleolar satellite during accelerated nucleoprotein synthesis,"Nature 163: 676, 1949.
10. Ohno, S., B.M. Cattanach, "Cytological study of an X-autosome translocation in Mus Musculus," Cytogenetics 1: 129, 1962.
11. Comings, D.E.,"Uridine-5-H^3 Radioautography of the Human Sex Chromatin Body," Journal of Cell Biology 28: 437, 1966.
12. Rao, S.R.V., V.K. Sharma, V.C.Shah,"DNA synthesis in duplicate type sex chromosomes of the Indian house shrew, Suncus Murinus (Insectivora)," Cytogenetics 9: 384, 1970.

13. Brown, S.W., "Heterochromatin," Science 151: 417, 1966.
14. Gartler, S.M., B.Burt, "Replication pattern of bovine sex chromosomes in cell culture," Cytogenetics 3: 135, 1964.
15. German, J., "DNA synthesis in human chromosomes," Trans. N.Y. Acad. Sci. 24: 395, 1962.
16. Grumbach, M.M., A. Morishima, J.H. Taylor, "Human sex chromosome abnormalities in relation to DNA replication and heterochromatization," Proc. Natl. Acad. Sci. USA. 49: 581, 1963.
17. Couturier, J., B. Dutrillaux, P. Garger, O. Raoul, M-F Croquette, J.C. Fourlinnie, E. Maillard, "Evidence for a correlation between late replication and autosomal gene inactivation in a familial translocation t(X;21)," Human Genetics 49: 319-326, 1979.
18. Zuffardi, O., L. Tiepolo, S. Scappaticci, D. Francesconi, C. Bianchi, D. DiNatali, "Reduced phenotypic effect of partial trisomy 1q in a X/1 translocation," Annales de Genetique, no. 3: 191-194, 1977.
19. Buhler, E.M., L.P. Jurik, M. Voyame, U.K. Buhler, "Presumptive evidence of two active X chromosomes in somatic cells of a human female," Nature 265: 142-144, 1977.
20. Bernstein, R., D.Bronwen, R. Kohl, T. Jenkins, "X-15 translocation in a retarded girl: X inactivation pattern and attempt to localize the hexosaminidase A and other loci," Journal of Medical Genetics 16: 254-262, 1979.
21. Eicher, E.M., "X-autosome translocations in the mouse: total inactivation versus partial inactivation of the X chromosome," Advances in Genetics 15: 175, 1970.
22. Muldal, S., C.W. Gilbert, L.G. Lajtha, J. Lindsten, J. Rowley, M. Fraccaro, "Tritiated thymidine incorporation in an isochromosome for the long arm of the X chromosome in man," Lancet 1963, i: 861.
23. Summitt, R., R.E. Tipton, R.S. Wilroy Jr.,,P.R. Martens, J.P. Phelan, "X-autosome translocations: a review," Birth Defects: Original Article Series Vol. XIV, no. 6C: 219-247, 1978.

24. Leisti, J.T., M.M. Kaback, D.L. Rimoin, "Human X-autosome translocations: differential inactivation of the X chromosome in a kindred with an X-9 translocation," Am. J. Hum. Genet. 27: 441-443, 1975.

25. Kallio, H., "Cytogenetic and clinical study of 100 cases of primary amenorrhea," Acta Obstet. Gynecol. Scand. (suppl.) 24: 1973.

26. Therman, E., K. Patau, "Abnormal X chromosomes in man: origin behavior, and effects," Humangenetik 25: 1-16, 1974.

27. Hagemeijer, A., J. Hoovers, E.M.S. Smit, D. Bootsma, "Replication pattern of the X chromosomes in three X/autosomal translocations," Cytogenet. Cell Genet. 18: 333-348, 1977.

28. Zabel, B.U., W.A. Bauman, W. Pirntke, K. Gerhard-Ratschow, "X-inactivation pattern in three cases of X/autosomal translocation," American Journal of Medical Genetics, 1: 309-317, 1978.

29. Fraccaro, M., P. Maraschio, F. Pasquali, S. Scappaticci, "Women heterozygous for deficiency of the p21-pter region of the X chromosome are fertile," Human Genetics 39: 283-292, 1977.

30. Thelen, T.H., D.J. Abrahms, R.O. Fisch, "Multiple abnormalities due to possible genetic inactivation in an X/autosome translocation," American Journal of Human Genetics 23: 410-418, 1971.

31. Mattei, M.G., J.F. Mattei, S. Ayme, G. Malpuech, F. Giraud, "A dynamic study in two new cases of X chromosome translocations," Human Genetics 41: 251-257, 1978.

32. Engel, W., W. Vogel, H. Reinwein, "Autoradiographische untersuchungen an einer X-Autosomentranslocation beim Menschen: 45,X, 15-, tan(15q;Xq+)," Cytogenetics 10: 87-98, 1971.

33. Gaal M., J. Laszlo, "X-inactivation in an unbalanced X-autosome translocation with gonadal dysgenesis," Human Heredity 27: 396-402, 1977.

34. Mikkelsen, M., G. Dahl, "Unbalanced X-autosomal translocation with inactivation of the normal X chromosome," Cytogenet. Cell Genet. 12: 357-366, 1973.

35. Cohen, M.M., C.C. Lin, V. Sybert, E.J. Orecchio, "Two human X-autosome translocations identified by autoradiography and fluorescence," American Journal of Human Genetics 24: 583-597, 1972.

36. Mann, J.D., J. Higgens, "A case of primary amenorrhea associated with X-autosomal translocation (46, X, t(Xq-;5q+))," American Journal of Human Genetics 26: 416, 1974.

37. Summitt, R., P.R. Martens, R.S. Wilroy Jr., "X-autosome translocation in normal mother and effectively 21-monosomic daughter," J. Pediatrics 84: 539-546, 1974.

38. Gilgenkrantz, S., G. Mauvary, B. Dutrillaux, G. Masocco, "Translocation X sur autosome et replication tardive," Humangenetik 26: 25-34, 1975.

39. Daly, R.F., K. Patau, E. Therman, T.E. Sarto, "Structure and Barr body formation of an Xp+ chromosome with two inactivation centers," Am. J. Human Genetics 29: 83-93, 1977.

40. Therman, E., G.E. Sarto, K. Patau, "Center for Barr body condensation on the proximal part of the human Xq: a hypothesis," Chromosoma 44: 361-366, 1974.

41. Cann, H.M., S.Sakaguchi, J. Stone, E.Gold, L.Luzzatti, "Familial X-autosome translocation (X;21)," Amer. J. Human Genetics 27: 23A, 1975.

42. Allderdice, P.W., O.J. Miller, H.P. Klinger, P.D. Pallister, J.M. Opitz, "Demonstration of a spreading effect in an X-autosome translocation by combined autoradiographic and quinacrine fluorescence studies (Abstr.)," Excerpta Med. Int. Congr. Ser. 233: 14-15, 1971.

43. Jenkins, M.B., E.Davis, T.H. Thelen,L. Boyd, "A familial X-22 translocation with an extra X chromosome," Amer. J. Human Genetics 26: 736-745, 1974.

44. Davis, J.R., M.W. Heine, E.S. Lightner, H.R. Giles, R.F. Graap, "X-short arm deletion gonadal dysgenesis in two siblings due to unique translocation (Xp-; 16p+)," Clin. Genet. 10: 202-207, 1976.

45. Disteche, C.M., E.M. Eicher, S.A. Latt, "Late Replication in an X-autosomal translocation in tne mouse: correlation with genetic inactivation and evidence for selective effects during embryogenesis," P.N.A.S., USA 76: 1979.

46. Latt, S.A., H.F. Willard, P.S. Gerald, "BrdU-33258 Hoechst analysis of DNA replication in human lymphocytes with supernumerary or structurally abnormal X chromosomes," Chromosoma (Berl) 57: 135-153, 1976.

47. Dutrillaux, B., L. Colette, S. Gilgenkrantz, J. Frederic, S. Carpentier, J. Couturier, et J. Lejeune, "Les translocations du chromosome X etude apres traitement par BUDR et coloration par l'acridine orange," Helv. Paediat. Acta Suppl. 34: 19-31, 1974.

48. Baker, W.K., "Position-effect variegation," Adv. Genet. 14: 133-169, 1968.

49. Russel, L.B., C.S. Montgomery, "Comparative studies on X-autosome translocations in the mouse II. Inactivation of autosomal loci, segregation, and mapping of autosomal breakpoints in five T(X;1)'s," Genetics 64: 281-212, Feb. 1970.

50. Crandall, B.F., R.E. Carrel, J. Howard, W.A. Schavener, "Trisomy with 13-X translocation," Amer. J. Hum. Genet. 26: 385-392, 1974.

51. Opitz, J., P.D. Pallister, "An (X;14) translocation unbalanced, 47 chromosomes, Repository identification no. GM 74," Cytogenet. Cell Genet. 12: 291-292, 1973.

53. Neuhauser, G., F.Back, "X-autosom-translokation bei einem kind mit multiplen missbildungen," Humangenetik 3: 300-301, 1967.

54. German, J., "Autoradiographic studies on human chromosomes," in Crow, J.F., J.V. Neel (eds.), Proceedings 3rd International Congress of Human Genetics, Baltimore, The Johns Hopkins Press, 1967.

55. Allderdice, P.W., O.J. Miller, D.A. Miller, H.P. Klinger, "Spreading of inactivation in an (X;14) translocation," Am. J. Medical Genetics 2: 233-240, 1978.

56. Murkerjee, D., W.J. Burdette, "Multiple congenital anomalies associated with a ring 3 chromosome and translocated 3/X chromosome," Nature 212: 153-155, 1966.

57. Tipton, R.E., "Cytogenetic studies in subjects with mental retardation and congenital anomalies using banding techniques," Master of Science Thesis, University of Tennessee, 1974.

58. Russel, L.B., "Mammalian X chromosome action: inactivation limited in spread and region of origin," Science 140: 976-978, 1963.

59. Therman, E., G.Sarto, C.G. Palmer, H. Kallio, C. Denniston, "Position of the human X inactivation center on Xq," Human Genetics 50: 59-64, 1979.

60. Buckton, K.E., P.A. Jacobs, L.A. Rae, M.S. Newton, R.Sanger, "An inherited X- autosome translocation in man," Ann. Hum. Genet. 35: 171-178, 1971.

61. Pallister, P.D., J.M. Opitz, "The KOP translocation," Birth Defects: Original Article Series, XIV no. 6C: 133-146, 1978.

62. Latt, S.A., "Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes," Proc. Nat. Acad. Sci. USA 70: 12: 3395-3399, 1973.

63. Willard, H.F., S.A. Latt, "Analysis of deoxyribonucleic acid replication in human X chromosomes by fluorescence microscopy," Am. J. Human Genetics 28: 213-227, 1976.

64. Miller, O.J. V.G. Dev, R. Tantravahi, D.A. Miller, "Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells," Exp. Cell Res. 101: 235-243, 1976.

65. Miller, D.A., R. Tantravahi, V.G. Dev, O.J. Miller, "Frequency of satellite association of human chromosomes is correlated with amount of Ag-staining of the nucleolus organizer region," Am. J. Hum. Genet. 29: 490-502, 1977.

66. Dutrillaux, B., J. Couturier, C-L. Richer, E. Vegas-Pequignot, "Sequence of DNA replication in 277 R and Q bands of human chromosomes using a BrdU treatment," Chromosoma (Berl.) 58: 51-61, 1976.

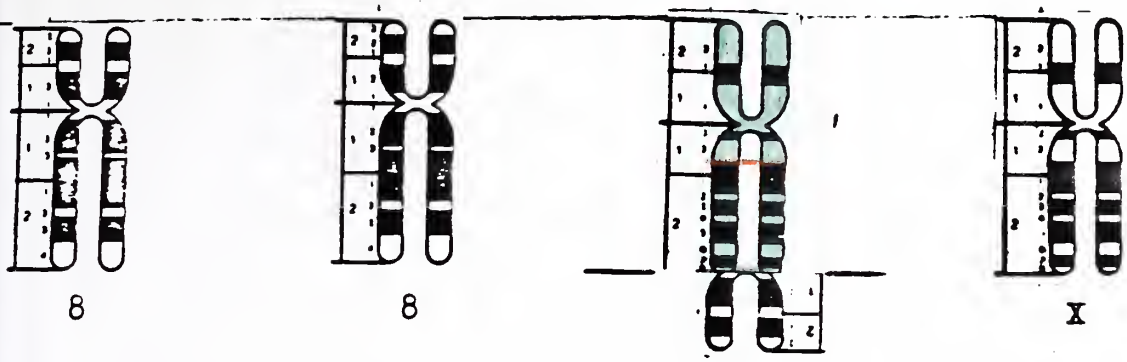
67. Pearson, E.S., C.J. Clapper, "The use of confidence or fiducial limits illustrated in the case of the binomial," Biometrika 26: 404, 1934.

68. Goodpasture, C., S.E. Bloom, "Visualization of nucleolus organizer regions in mammalian chromosomes using silver stain," Chromosoma 53: 37-50, 1975.

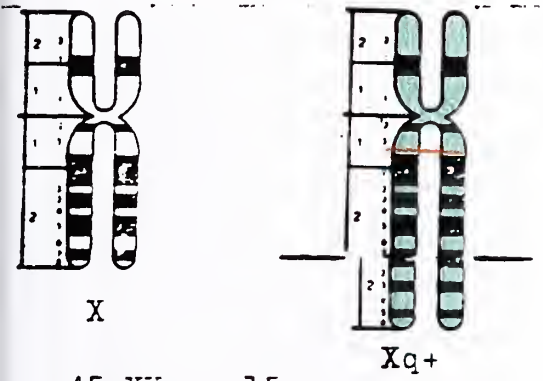
69. Miller, O.J., D.A. Miller, R.E. Kouri, P. Allderdice, V.G. Dev, M.S. Grewal, J.J. Hutton, "Identification of the Mouse karyotype by quinacrine fluorescence and tentative assignment of seven linkage groups", Proc. Natl. Acad. Sci. USA 68: 1530, 1971.

APPENDIX

Spreading patterns in known X-autosome translocations. All reported unbalanced translocations are shown as are those balanced translocations in which there is inactivation of a translocation chromosome. Green regions are regions of the translocation chromosome(s) in which spreading of inactivation always occurs. Purple regions are areas in which spreading is variable. Horizontal red lines indicate the proposed inactivation center on proximal Xq.

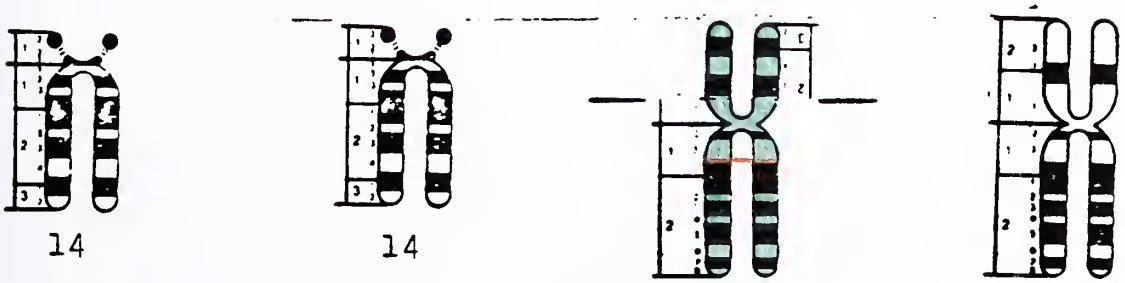


46,X,der(X),t(X;8)(q28?;q11?)
Cohen

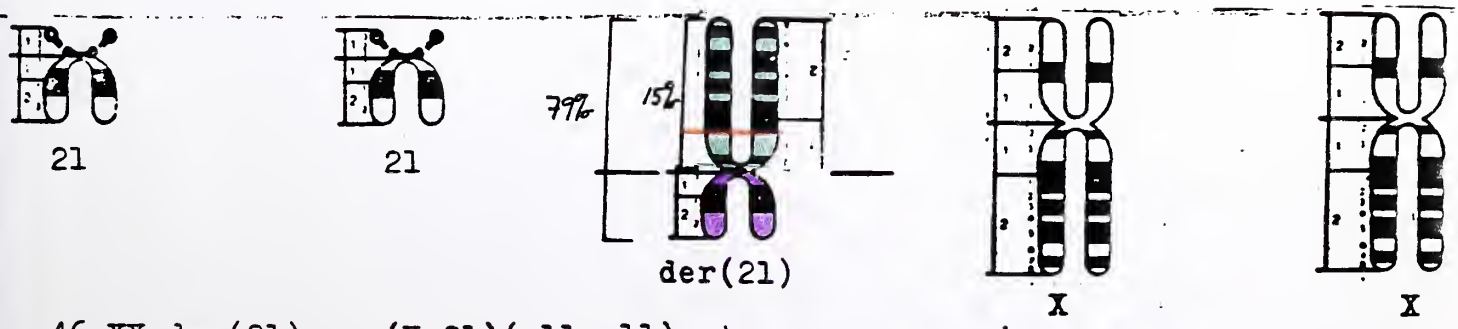


Entire X_t late replicating – 75% of cells
Normal X 11%
Both 10%

45,XXq+,-15
Engel

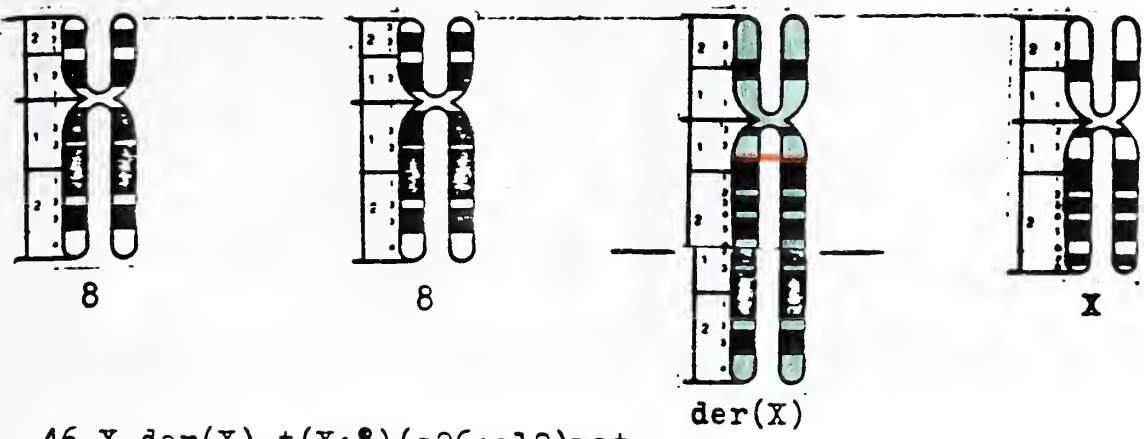


45,X/46,X,der(X),t(Xp-;14q+)
Buckton

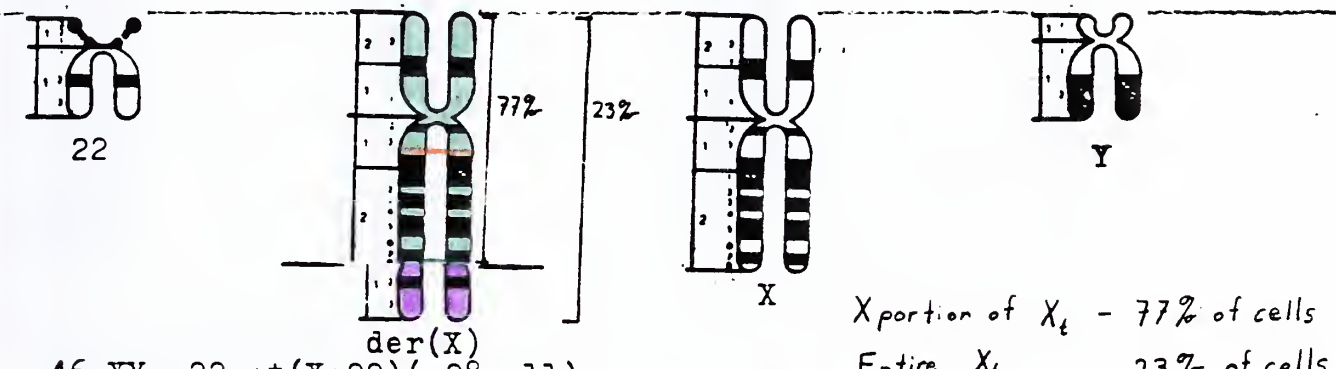


46,XX,der(21),rcp(X;21)(q11;p11)mat
Summitt

Normal X – 100% of cells
Normal X and entire X_t 79%
Normal X and X portion of X_t 15%

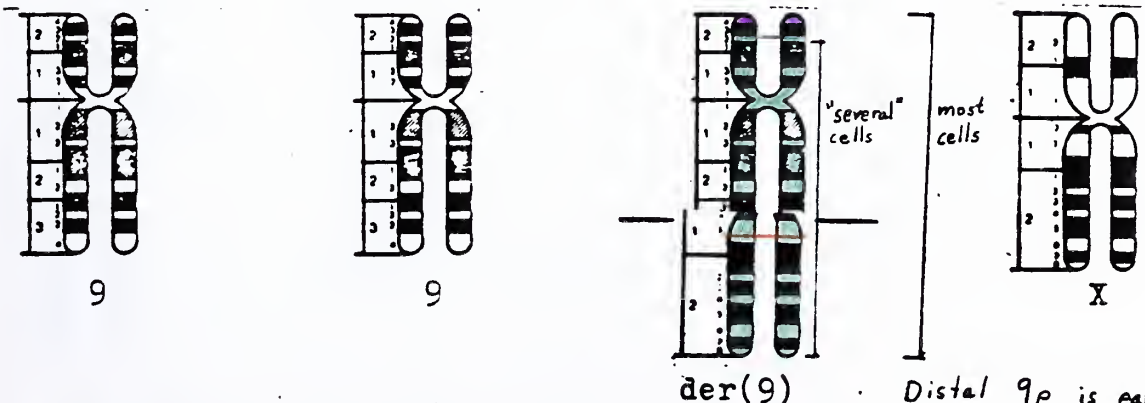


46,X,der(X),t(X;8)(q26;q12)mat
Tipton



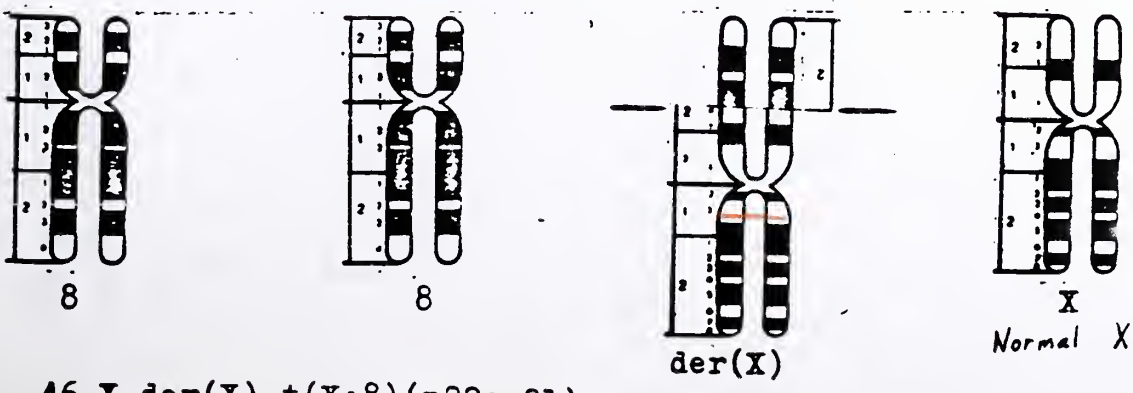
X portion of X_t - 77% of cells
Entire X_t 23% of cells

46,XY,-22,+t(X;22)(q28;q11)
Jenkins



Distal 9p is early replicating only in "several" cells

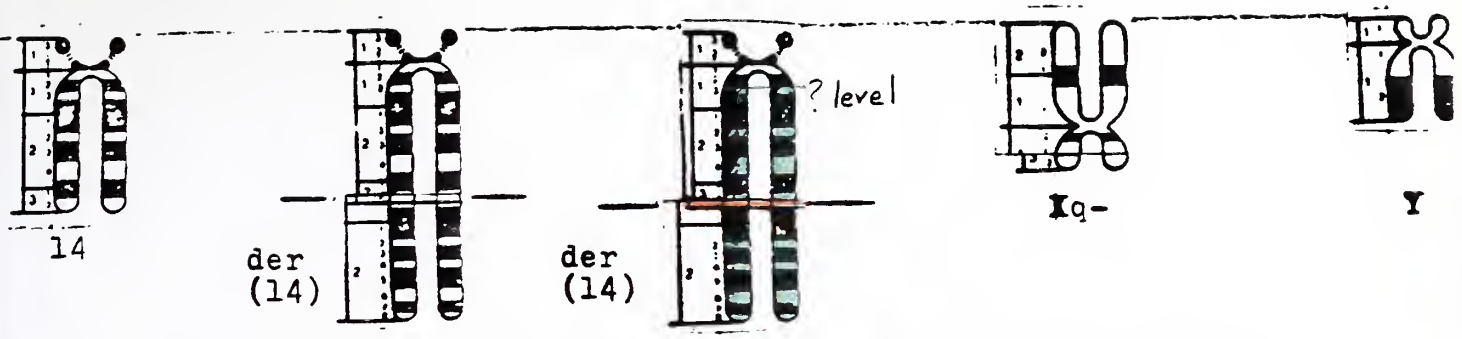
46,X,-X,+der(9),rcp(X;9)(q11;q32)mat
Leisti



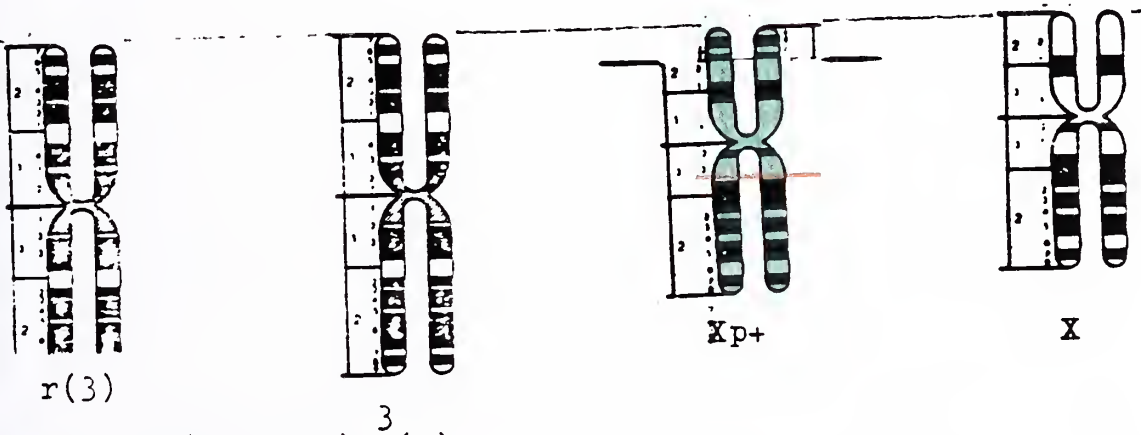
Normal X - 100% of cells

46,X,der(X),t(X;8)(p22;q21)

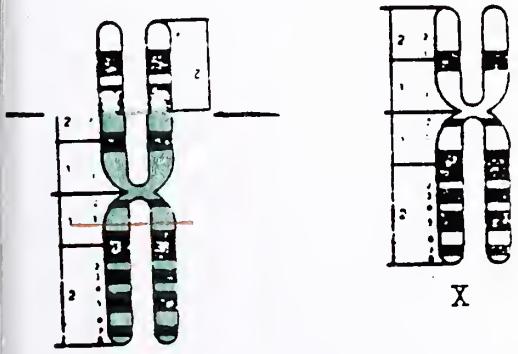
Mikkelsen



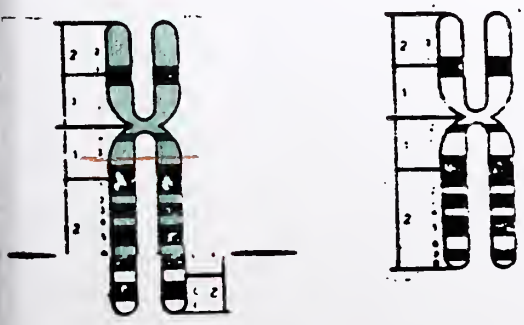
47, Xq-,Y, der(14), t(X;14)(q13;q32), +der(14),(X;14)(q13;q32)
Allderdice



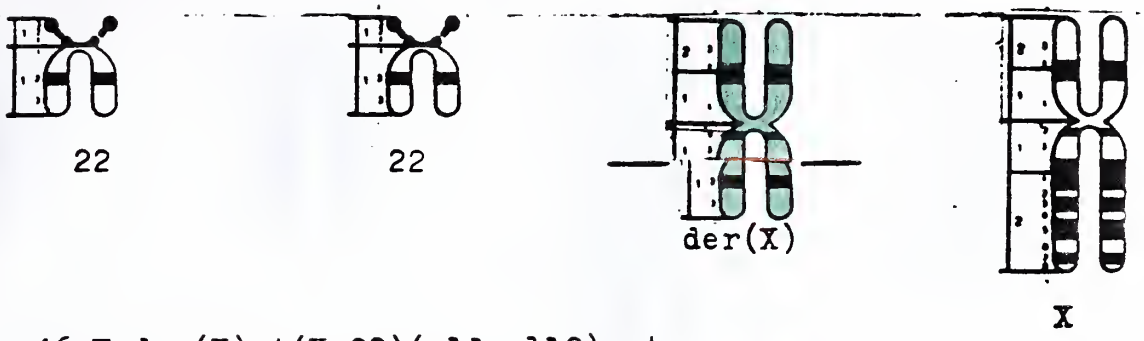
46, X,t(Xp+;3q-),r(3)
Mukerjee



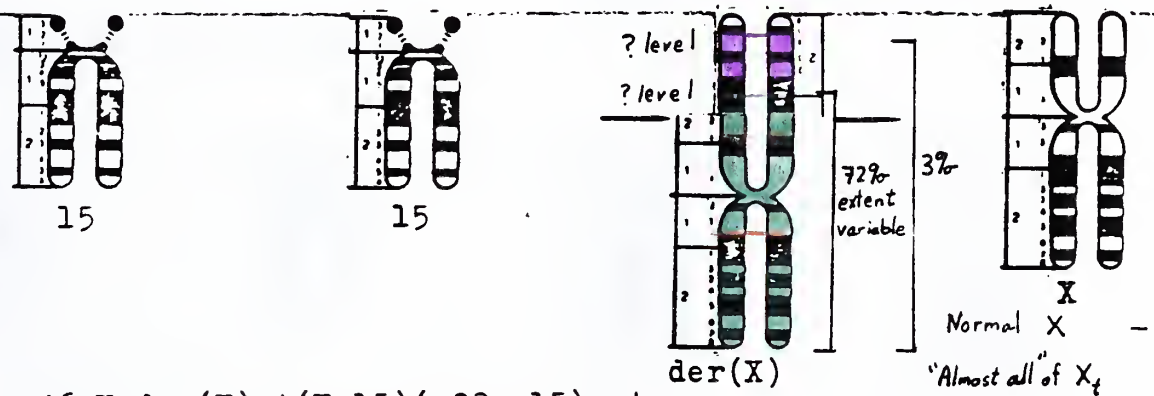
45,XXp+, -C
Neuhauser



46, XXq+ German

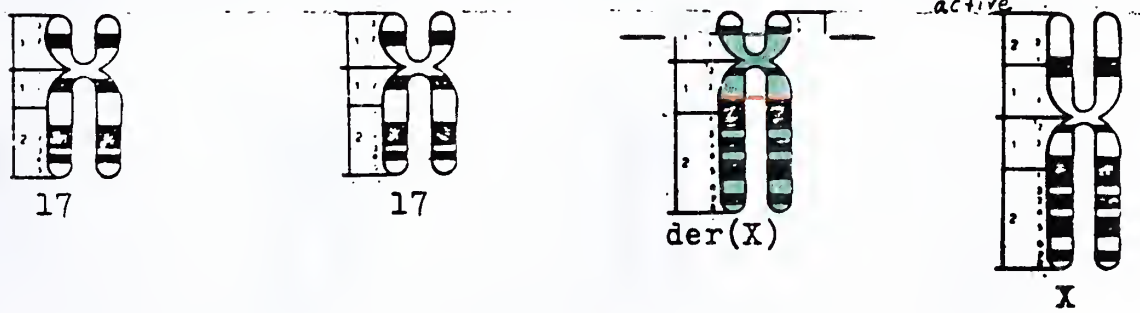


46,X,der(X),t(X,22)(q13,q112)mat
Mattei

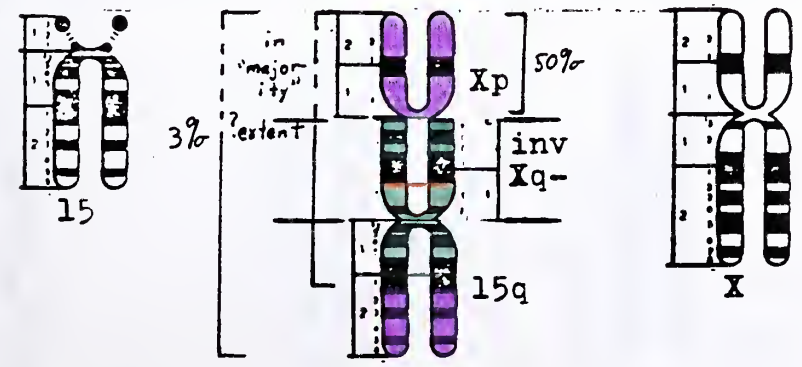


Normal X — 25% of cells
"Almost all" of X_t 3%
X_t inactive major part of autosome active 72%

46,X,der(X),t(X;15)(p22,q15)mat
Zabel



46,X,der(X),t(X;17)(p11;q24)mat
Hagemeijer



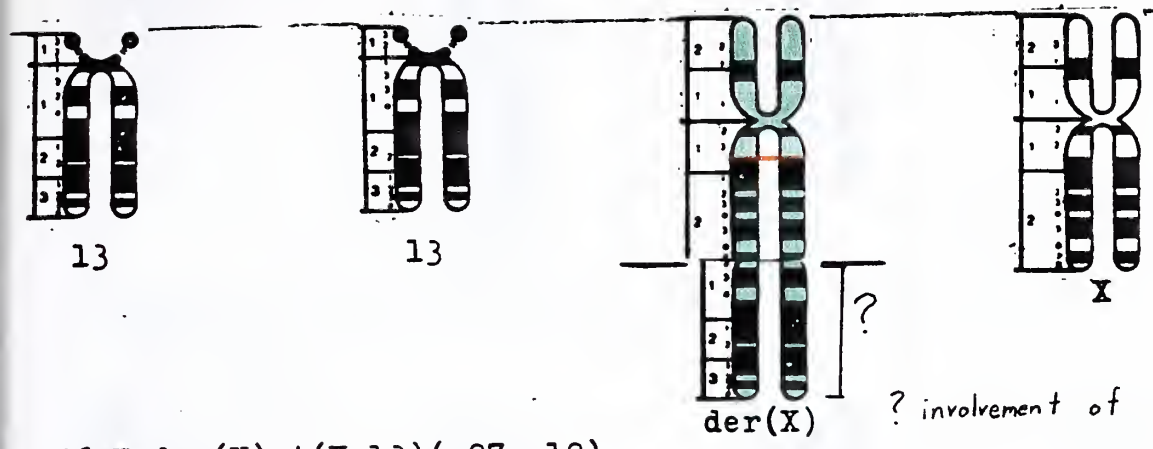
45,X,-15,+t(X;15) -- see above
Bernstein

X_t always the inactive chromosome

Spread in majority of cells ?level, but not to 15q ter

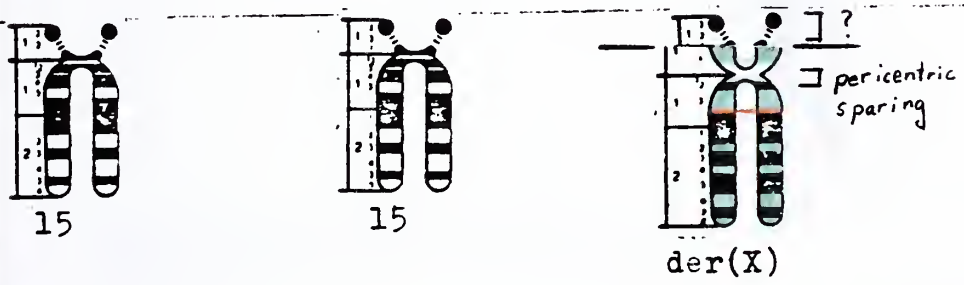
Entire X_t in 3% of cells

NB: X_p inactive in only 50% of cells



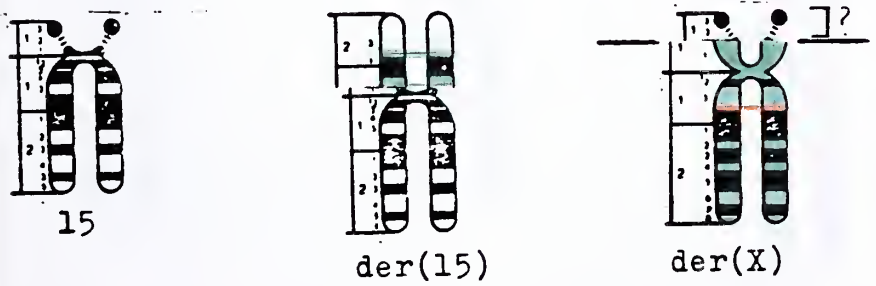
? involvement of 13 portion of X_t

46,X,der(X),t(X;13)(q27;q12)
Crandall



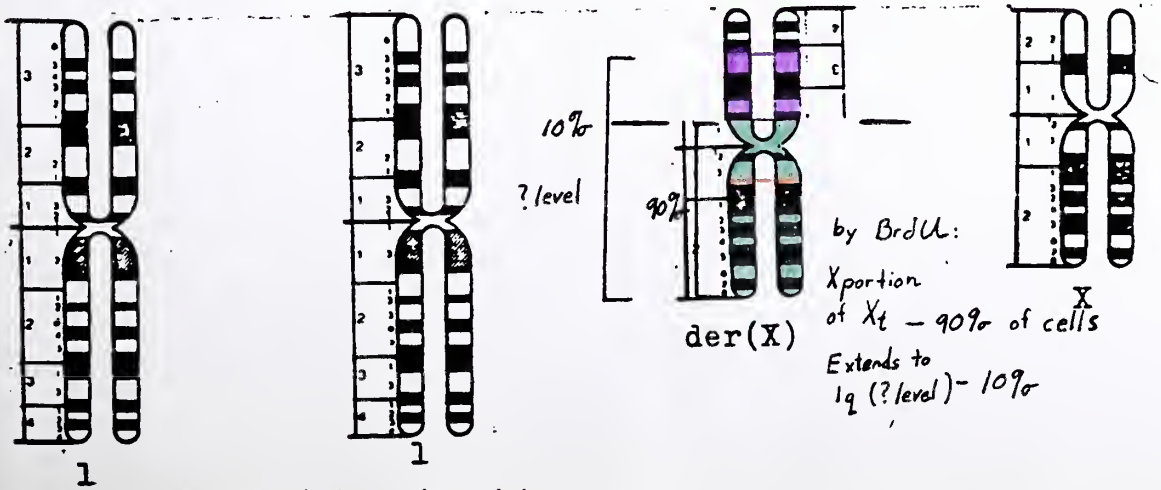
46,X,der(X),t(X;15)(15pter-15p1::Xp113-Xqter;Xpter-Xp113::15p1-15qter)mat
Fraccaro

} Same family



Normal X - 62% of cells
Der(X)
(?15 portion)
⊕ $Xp113 - Xp22$ - 38% of der(15)

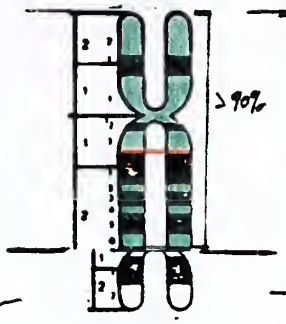
46,X,t(X;15)(15pter-15p1::Xp113-Xqter;Xpter-Xp113::15p1-15qter)
Fraccaro



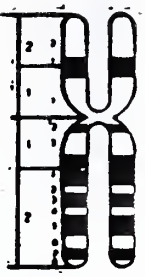
by BrdU:
X portion of X_t - 90% of cells
Extends to 1q (?level) - 10%

46,X,-X,+der(X), t(X;1)(Xqter-Xp11::1q24-1qter) Zuffardi

21



der(X)



X

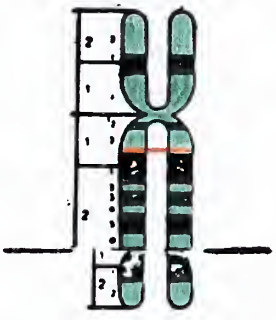
X portion of X_t >90% of cells

45,X,-21,der(X),t(X;21)(q2700;q11) Spread to sub-band 21q221 < 10%
Couturier

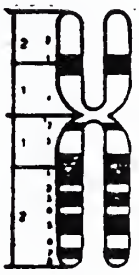
Same family

21

21



der(X)

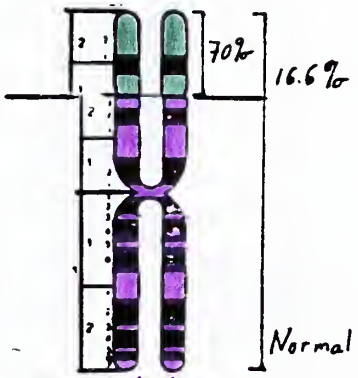


X

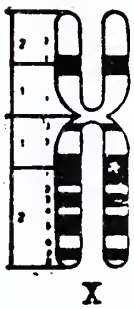
46,X,der(X),t(X;21)(q2700;q11)
Couturier

6

6



der(6)



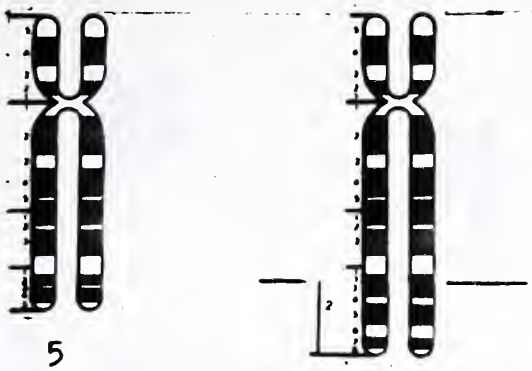
X

Entire der(6) 16.6%

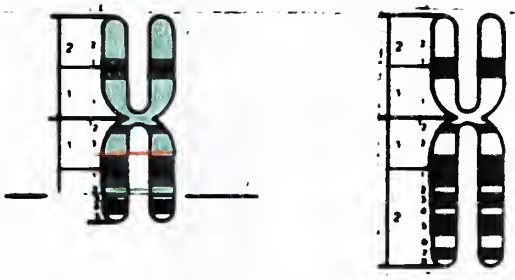
X portion of der(6) 70%

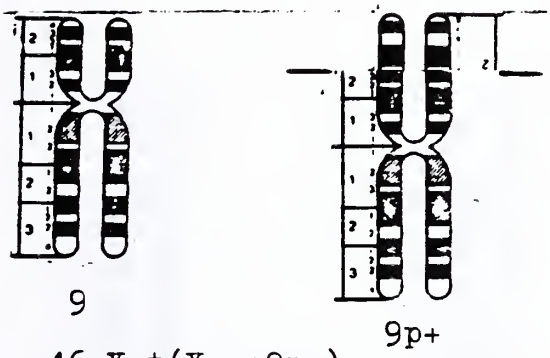
13.4% of cells

46,X,-X,+der(6),t(X;6)(p21,;24)mat
Gaal

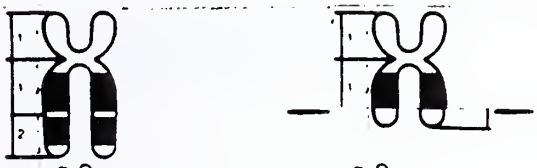


46,X,t(Xq-,5q+)
Mann

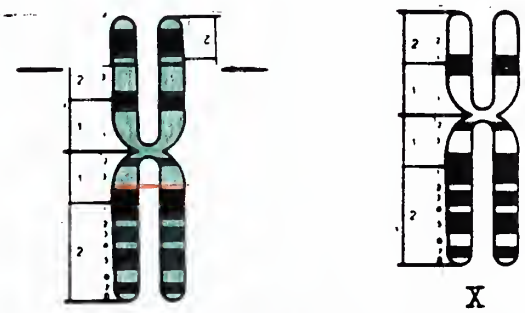


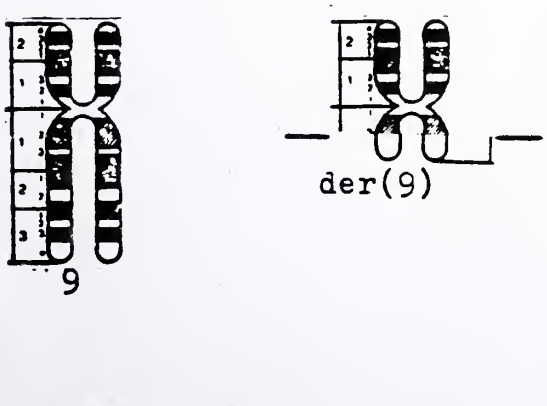


46,X,t(Xq-;9p+)
Cohen

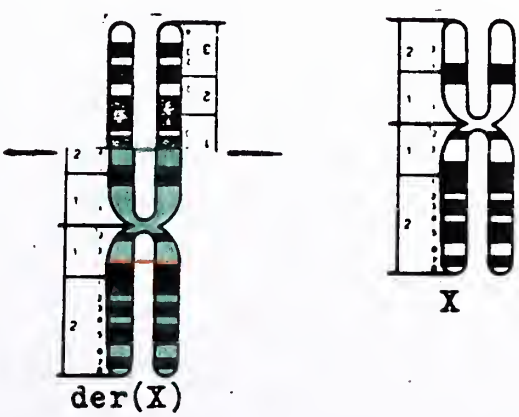


46,X,t(Xp+;18q-)
Thelen

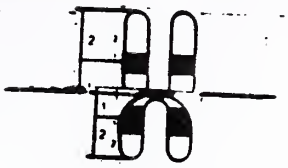




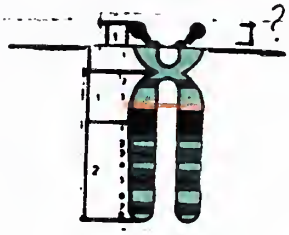
46,X,t(9;X)(q12,p22)
Mattei



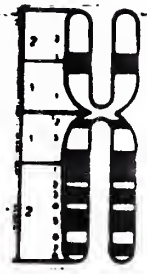
21



der(21)



der(X)



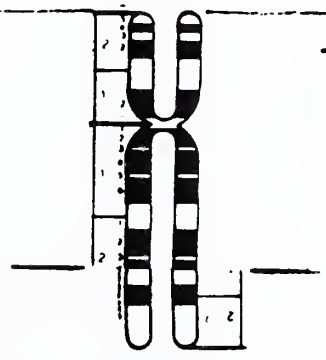
X

46,X,t(X;21)(p11;p11?)
Zabel

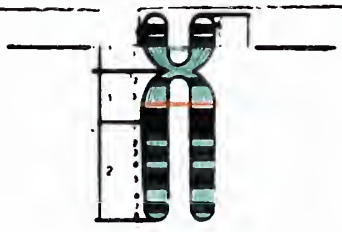
Normal X 70% of cells

$X_p^{\ominus}$ 30%

6



der(6)



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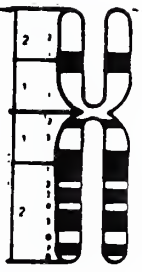

X

46,X.t(X;6)(p21,q26)
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